Peter, Birk [DK/DK]; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). JENSEN, Martin, Roland [DK/DK]; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). NIELSEN, Klaus, Gregorius [DK/DK]; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). KOEFOED, Peter [DK/DK]; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). DEGAN, Florence, Dal [DZ/DZ]; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK).

(74) Agent: KOEFOED, Peter; Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK).

[Continued on next page]

(54) Title: NOVEL METHOD FOR DOWN-REGULATION OF AMYLOID

PEPCoVAC SYNTHESIS

peptide A

peptide B

tresyl activated dextran, TAD

+ → Purification
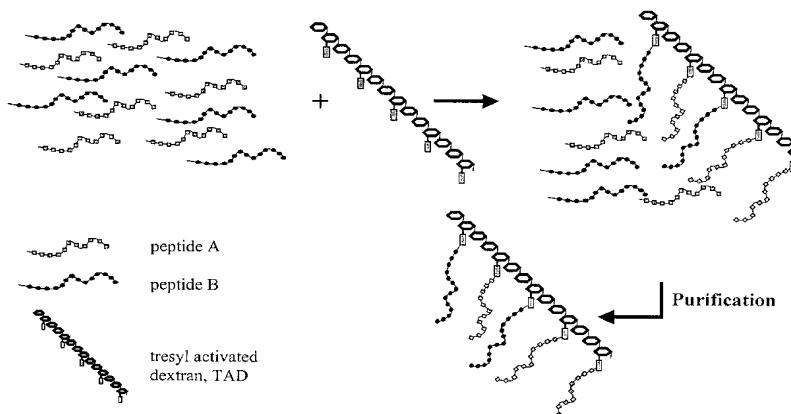
(57) Abstract: Disclosed are novel methods for combatting diseases characterized by deposition of amyloid. The methods generally rely on immunization against amyloid precursor protein (APP) or beta amyloid (Aβ). Immunization is preferably effected by administration of analogues of autologous APP or Aβ, said analogues being capable of inducing antibody production against the autologous amyloidogenic polypeptides. Especially preferred as an immunogen is autologous Aβ which has been modified by introduction of one single or a few foreign, immunodominant and promiscuous T-cell epitopes. Also disclosed are nucleic acid vaccination against APP or Aβ and vaccination using live vaccines as well as methods and means useful for the vaccination. Such methods and means include methods for the preparation of analogues and pharmaceutical formulations, as well as nucleic acid fragments, vectors, transformed cells, polypeptides and pharmaceutical formulations.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

NOVEL METHOD FOR DOWN-REGULATION OF AMYLOID

FIELD OF THE INVENTION

The present invention relates to improvements in therapy and
prevention of Alzheimer's disease (AD) and other diseases
5   characterized by deposition of amyloid, e.g. characterized by
amyloid deposits in the central nervous system (CNS). More
specifically, the present invention provides a method for
down-regulating (undesired) deposits of amyloid by enabling
the production of antibodies against a relevant protein (APP
10   or Aβ) or components thereof in subjects suffering from or in
danger of suffering from diseases having a pathology involving
amyloid deposition. The invention also provides for methods of
producing polypeptides useful in this method as well as for
the modified polypeptides as such. Also encompassed by the
15   present invention are nucleic acid fragments encoding the
modified polypeptides as well as vectors incorporating these
nucleic acid fragments and host cells and cell lines trans-
formed therewith. Finally, the present invention also provides
for a new type of conjugate peptide immunogen.

20   BACKGROUND OF THE INVENTION

Amyloidosis is the extracellular deposition of insoluble pro-
tein fibrils leading to tissue damage and disease (Pepys,
1996; Tan et al., 1995; Kelly, 1996). The fibrils form when
normally soluble proteins and peptides self-associate in an
25   abnormal manner (Kelly, 1997).

Amyloid is associated with serious diseases including systemic
amyloidosis, AD, maturity onset diabetes, Parkinson's disease,
Huntington's disease, fronto-temporal dementia and the

prion-related transmissible spongiform encephalopathies (kuru and Creutzfeldt-Jacob disease in humans and scrapie and BSE in sheep and cattle, respectively) and the amyloid plaque formation in for instance Alzheimer's seems to be closely associ-

5   ated with the progression of human disease. In animal models over-expression, or the expression of modified forms, of proteins found in deposits, like the β-amyloid protein, has been shown to induce various symptoms of disease, e.g. Alzheimer's-like symptoms. There is no specific treatment for amyloid

10 deposition and these diseases are usually fatal.

The subunits of amyloid fibrils may be wild-type, variant or truncated proteins, and similar fibrils can be formed in vitro from oligopeptides and denatured proteins (Bradbury et al., 1960; Filshie et al., 1964; Burke & Rougvie, 1972). The nature

15 of the polypeptide component of the fibrils defines the character of the amyloidosis. Despite large differences in the size, native structure and function of amyloid proteins, all amyloid fibrils are of indeterminate length, unbranched, 70 to 120 Å in diameter, and display characteristic staining with

20 Congo Red (Pepys, 1996). They are characteristic of a cross-β structure (Pauling & Corey, 1951) in which the polypeptide chain is organized in β-sheets. Although the amyloid proteins have very different precursor structures, they can all undergo a structural conversion, perhaps along a similar pathway, to a

25 misfolded form that is the building block of the β-sheet helix protofilament.

This distinctive fibre pattern led to the amyloidoses being called the β-fibrilloses (Glenner, 1980a,b), and the fibril protein of AD was named the β-protein before its secondary

30 structure was known (Glenner & Wong, 1984). The characteristic

cross-β diffraction pattern, together with the fibril appear-
ance and tinctorial properties are now the accepted diagnostic
hallmarks of amyloid, and suggest that the fibrils, although
formed from quite different protein precursors, share a degree
of structural similarity and comprise a structural super-
family, irrespective of the nature of their precursor proteins
(Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake
CCFJ Mol Biol 1997 Oct 31; 273(3):729-739).

One of the most widespread and well-known diseases where amy-
loid deposits in the central nervus system are suggested to
have a central role in the progression of the disease, is AD.

AD

Alzheimer's disease (AD) is an irreversible, progressive brain
disorder that occurs gradually and results in memory loss, be-
havioural and personality changes, and a decline in mental
abilities. These losses are related to the death of brain
cells and the breakdown of the connections between them. The
course of this disease varies from person to person, as does
the rate of decline. On average, AD patients live for 8 to 10
years after they are diagnosed, though the disease can last
for up to 20 years.

AD advances by stages, from early, mild forgetfulness to a se-
vere loss of mental function. This loss is known as dementia.
In most people with AD, symptoms first appear after the age of
60, but earlier onsets are not infrequent. The earliest symp-
toms often include loss of recent memory, faulty judgment, and
changes in personality. Often, people in the initial stages of
AD think less clearly and forget the names of familiar people
and common objects. Later in the disease, they may forget how
to do even simple tasks. Eventually, people with AD lose all

reasoning ability and become dependent on other people for
their everyday care. Ultimately, the disease becomes so de-
bilitating that patients are bedridden and likely to develop
other illnesses and infections. Most commonly, people with AD

5   die from pneumonia.

Although the risk of developing AD increases with age, AD and
dementia symptoms are not a part of normal aging. AD and other
dementing disorders are caused by diseases that affect the
brain. In normal aging, nerve cells in the brain are not lost

10 in large numbers. In contrast, AD disrupts three key pro-
cesses: Nerve cell communication, metabolism, and repair. This
disruption ultimately causes many nerve cells to stop func-
tioning, lose connections with other nerve cells, and die.

At first, AD destroys neurons in parts of the brain that con-

15 trol memory, especially in the hippocampus and related struc-
tures. As nerve cells in the hippocampus stop functioning
properly, short-term memory fails, and often, a person's abi-
lity to do easy and familiar tasks begins to decline. AD also
attacks the cerebral cortex, particularly the areas responsi-

20 ble for language and reasoning. Eventually, many other areas
of the brain are involved, all these brain regions atrophy
(shrink), and the AD patient becomes bedridden, incontinent,
totally helpless, and unresponsive to the outside world
(source: National Institute on Aging Progress Report on Alz-

25 heimer's Disease, 1999).

The Impact of AD

AD is the most common cause of dementia among people age 65
and older. It presents a major health problem because of its
enormous impact on individuals, families, the health care sys-

30 tem, and society as a whole. Scientists estimate that up to 4

million people currently suffer from the disease, and the prevalence doubles every 5 years beyond age 65. It is also estimated that approximately 360,000 new cases (incidence) will occur each year, though this number will increase as the popu-
5   lation ages (Brookmeyer et al., 1998).

AD puts a heavy economic burden on society. A recent study in the United States estimated that the annual cost of caring for one AD patient is $18,408 for a patient with mild AD, $30,096 for a patient with moderate AD, and $36,132 for a patient with
10 severe AD. The annual national cost of caring for AD patients in the US is estimated to be slightly over $50 billion (Leon et al., 1998).

Approximately 4 million Americans are 85 or older, and in most industrialized countries, this age group is one of the fastest
15 growing segments of the population. It is estimated that this group will number nearly 8.5 million by the year 2030 in the US; some experts who study population trends suggest that the number could be even greater. As more and more people live longer, the number of people affected by diseases of aging,
20 including AD, will continue to grow. For example, some studies show that nearly half of all people age 85 and older have some form of dementia. (National Institute on Aging Progress Report on Alzheimer's Disease, 1999)

The Main Characteristics of AD

25 Two abnormal structures in the brain are the hallmarks of AD: amyloid plaques and neurofibrillary tangles (NFT). Plaques are dense, largely insoluble deposits of protein and cellular material outside and around the brain's neurons. Tangles are insoluble twisted fibres that build up inside neurons.

6

Two types of AD exist: familial AD (FAD), which follows a certain pattern of inheritance, and sporadic AD, where no obvious pattern of inheritance is seen. Because of differences in the age at onset, AD is further described as early-onset (occur-
5   ring in people younger than 65) or late-onset (occurring in those 65 and older). Early-onset AD is rare (about 10 percent of cases) and generally affects people aged 30 to 60. Some forms of early-onset AD are inherited and run in families. Early-onset AD also often progresses faster than the more com-
10   mon, late-onset form.

All FADs known so far have an early onset, and as many as 50 percent of FAD cases are now known to be caused by defects in three genes located on three different chromosomes. These are mutations in the APP gene on chromosome 21; mutations in a
15   gene on chromosome 14, called presenilin 1; and mutations in a gene on chromosome 1, called presenilin 2. There is as yet no evidence, however, that any of these mutations play a major role in the more common, sporadic or non-familial form of late-onset AD. (National Institute on Aging Progress Report on
20   Alzheimer's Disease, 1999)

Amyloid Plaques

In AD, amyloid plaques develop first in areas of the brain used for memory and other cognitive functions. They consist of largely insoluble deposits of beta amyloid (hereinafter desig-
25   nated $A\beta$) – a protein fragment of a larger protein called amyloid precursor protein (APP, the amino acid sequence of which is set forth in SEQ ID NO: 2) – intermingled with portions of neurons and with non-nerve cells such as microglia and astrocytes. It is not known whether amyloid plaques themselves
30   constitute the main cause of AD or whether they are a

7

by-product of the AD process. Certainly, changes in the APP protein can cause AD, as shown in the inherited form of AD caused by mutations in the APP gene, and Aβ plaque formation seems to be closely associated with the progression of the hu-
5 man disease (Lippa C. F. *et al.* 1998).

APP

APP is one of many proteins that are associated with cell membranes. After it is made, APP becomes embedded in the nerve cell's membrane, partly inside and partly outside the cell.
10 Recent studies using transgenic mice demonstrate that APP appears to play an important role in the growth and survival of neurons. For example, certain forms and amounts of APP may protect neurons against both short- and long-term damage and may render damaged neurons better able to repair themselves
15 and help parts of neurons grow after brain injury.

While APP is embedded in the cell membrane, proteases act on particular sites in APP, cleaving it into protein fragments. One protease helps cleave APP to form Aβ, and another protease cleaves APP in the middle of the amyloid fragment so that Aβ
20 cannot be formed. The Aβ formed is of two different lengths, a shorter 40 (or 41) amino acids Aβ that is relatively soluble and aggregates slowly, and a slightly longer, 42 amino acids "sticky" Aβ that rapidly forms insoluble clumps. While Aβ is being formed, it is not yet known exactly how it moves through
25 or around nerve cells. In the final stages of this process, the "sticky" Aβ aggregates into long filaments outside the cell and, along with fragments of dead and dying neurons and the microglia and astrocytes, forms the plaques that are characteristic of AD in brain tissue.

Some evidence exists that the mutations in APP render more likely that Aβ will be snipped out of the APP precursor, thus causing either more total Aβ or relatively more of the "sticky" form to be made. It also appears that mutations in the

5   presenilin genes may contribute to the degeneration of neurons in at least two ways: By modifying Aβ production or by trigger- ing the death of cells more directly. Other researchers sug- gest that mutated presenilins 1 and 2 may be involved in ac- celerating the pace of apoptosis.

10 It is to be expected that as the disease progresses, more and more plaques will be formed, filling more and more of the brain. Studies suggest that it may be that the Aβ is aggrega- ting and disaggregating at the same time, in a sort of dynamic equilibrium. This raises the hope that it may be possible to

15 break down the plaques even after they have formed. (National Institute on Aging Progress Report on Alzheimer's Disease, 1999).

It is believed that Aβ is toxic to neurons. In tissue culture studies, researchers observed an increase in death of

20 hippocampal neurons cells engineered to over-express mutated forms of human APP compared to neurons over-expressing the normal human APP (Luo *et al.*, 1999).

Furthermore, overexpression or the expression of modified forms of the Aβ protein has in animal models been demonstrated

25 to induce Alzheimer-like symptoms, (Hsiao K. *et al.*, 1998)

Given that increased Aβ generation, its aggregation into pla- ques, and the resulting neurotoxicity may lead to AD, it is of therapeutic interest to investigate conditions under which Aβ aggregation into plaques might be slowed down or even blocked.

Presenilins

Mutations in presenilin-1 (S-180) account for almost 50% of
all cases of early-onset familial AD (FAD). Around 30 muta-
tions have been identified that give rise to AD. The onset of
5   AD varies with the mutations. Mutations in presenilin-2 ac-
count for a much smaller part of the cases of FAD, but is
still a significant factor. It is not known whether prese-
nilins are involved in sporadic non-familial AD. The function
of the presenilins is not known, but they appear to be in-
10 volved in the processing of APP to give Aβ-42 (the longer
stickier form of the peptide, SEQ ID NO: 2, residues 673-714),
since AD patients with presenilin mutations have increased
levels of this peptide. It is unclear whether the presenilins
also have a role in causing the generation of NFT's. Some sug-
15 gest that presenilins could also have a more direct role in
the degeneration of neurons and neuron death. Presenilin-1 is
located at chromosome 14 while presenilin-2 is linked to chro-
mosome 1. If a person harbours a mutated version of just one
of these genes he or she is almost certain to develop early
20 onset AD.

There is some uncertainty to whether presenilin-1 is identical
to the hypothetical gamma-secretase involved in the processing
of APP (Naruse et al., 1998).

Apolipoprotein E

25 Apolipoprotein E is usually associated with cholesterol, but
is also found in plaques and tangles of AD brains. While al-
leles 1-3 do not seem to be involved in AD there is a signifi-
cant correlation between the presence of the APOE-ε4 allele
and development of late AD (Strittmatter et al., 1993). It is,
30 however, a risk factor and not a direct cause as is the case

for the presenilin and APP mutations and it is not limited to familial AD.

The ways in which the ApoE ε4 protein increases the likelihood of developing AD are not known with certainty, but one possi-
5   ble theory is that it facilitates Aβ buildup and this contri-butes to lowering the age of onset of AD, or the presence or absence of particular APOE alleles may affect the way neurons respond to injury (Buttini et al., 1999).

Also Apo A1 has been shown to be amyloigenic. Intact apo A1
10 can itself form amyloid-like fibrils *in vitro* that are Congo red positive (Am J Pathol 147 (2): 238-244 (Aug 1995), Wisniewski T, Golabek AA, Kida E, Wisniewski KE, Frangione B).

There seem to be some contradictory results indicating that there is a positive effect of the APOE-ε4 allele in decreasing
15 symptoms of mental loss, compared to other alleles (Stern, Brandt, 1997, Annals of Neurology 41).

Neurofibrillary Tangles

This second hallmark of AD consists of abnormal collections of twisted threads found inside nerve cells. The chief component
20 of tangles is one form of a protein called tau (τ). In the central nervous system, tau proteins are best known for their ability to bind and help stabilize microtubules, which are one constituent of the cell's internal support structure, or skeleton. However, in AD tau is changed chemically, and this
25 altered tau can no longer stabilize the microtubules, causing them to fall disintegrate. This collapse of the transport sys-tem may at first result in malfunctions in communication be-tween nerve cells and may later lead to neuronal death.

In AD, chemically altered tau twists into paired helical fila-
ments - two threads of tau that are wound around each other.
These filaments are the major substance found in neurofibri-
llary tangles. In one recent study, researchers found neurofi-
5   brillary changes in fewer than 6 percent of the neurons in a
particular part of the hippocampus in healthy brains, in more
than 43 percent of these neurons in people who died with mild
AD, and in 71 percent of these neurons in people who died with
severe AD. When the loss of neurons was studied, a similar
10 progression was found. Evidence of this type supports the idea
that the formation of tangles and the loss of neurons progress
together over the course of AD. (National Institute on Aging
Progress Report on Alzheimer's Disease, 1999).

Tauopathies and Tangles

15 Several neurodegenerative diseases, other than AD, are charac-
terized by the aggregation of tau into insoluble filaments in
neurons and glia, leading to dysfunction and death. Very re-
cently, several groups of researchers, who were studying fami-
lies with a variety of hereditary dementias other than AD,
20 found the first mutations in the tau gene on chromosome 17
(Clark *et al.*, 1998; Hutton *et al.*, 1998; Poorkaj *et al.*,
1998; Spillantini *et al.*, 1998). In these families, mutations
in the tau gene cause neuronal cell death and dementia. These
disorders which share some characteristics with AD but differ
25 in several important respects, are collectively called "fronto
temporal dementia and parkinsonism linked to chromosome 17"
(FTDP-17). They are diseases such as Parkinson's disease, some
forms of amyotrophic lateral sclerosis (ALS), corticobasal de-
generation, progressive supranuclear palsy, and Pick's dis-
30 ease, and are all characterized by abnormal aggregation of tau
protein.

12

Other AD-like neurological diseases.

There are important parallels between AD and other neurologi-
cal diseases, including prion diseases (such as kuru,
Creutzfeld-Jacob disease and bovine spongiform encephalitis),
5   Parkinson's disease, Huntington's disease, and fronto-temporal
dementia. All involve deposits of abnormal proteins in the
brain. AD and prion diseases cause dementia and death, and
both are associated with the formation of insoluble amyloid
fibrils, but from membrane proteins that are different from
10 each other.

Scientists studying Parkinson's disease, the second most com-
mon neurodegenerative disorder after AD, discovered the first
gene linked to the disease. This gene codes for a protein
called synuclein, which, intriguingly, is also found in the
15 amyloid plaques of AD patients' brains (Lavedan C, 1998, Ge-
nome Res. 8(9): 871-80). Investigators have also discovered
that genetic defects in Huntington's disease, another progres-
sive neurodegenerative disorder that causes dementia, cause
the Huntington protein to form into insoluble fibrils very
20 similar to the Aβ fibrils of AD and the protein fibrils of
prion disease, (Scherzinger E, et al., 1999, PNAS U.S.A.
96(8): 4604-9).

Scientists have also discovered a novel gene, which when mu-
tated, is responsible for familial British dementia (FBD), a
25 rare inherited disease that causes severe movement disorders
and progressive dementia similar to that seen in AD. In a bio-
chemical analysis of the amyloid fibrils found in the FBD
plaques, a unique peptide named ABri was found (Vidal et al.,
1999). A mutation at a particular point along this gene re-
30 sults in the production of a longer-than-normal Bri protein.

The ABri peptide, which is snipped from the mutated end of the Bri protein is deposited as amyloid fibrils. These plaques are thought to lead to the neuronal dysfunction and dementia that characterizes FBD.

5 **Immunization with Aβ**

The immune system will normally take part in the clearing of foreign protein and proteinaceous particles in the organism but the deposits associated with the above-mentioned diseases consist mainly of self-proteins, thereby rendering the role of

10 the immune system in the control of these diseases less obvious. Further, the deposits are located in a compartment (the CNS) normally separated from the immune system, both facts suggesting that any vaccine or immunotherapeutical approach would be unsuccessful.

15 Nevertheless, scientists have recently attempted immunizing mice with a vaccine composed of heterologous human Aβ and a substance known to excite the immune system (Schenk *et al.*, 1999 and WO 99/27944). The vaccine was tested in a partial transgenic mouse model of AD with a human mutated gene for APP

20 inserted into the DNA of the mouse. The mice produced the modified APP protein and developed amyloid plaques as they grew older. This mouse model was used to test whether vaccination against the modified transgenic *human* APP had an effect on plaque build-up. In a first experiment, one group of trans-

25 genic mice was given monthly injections of the vaccine starting at 6 weeks of age and ending at 11 months. A second group of transgenic mice received no injections and served as a control group. By 13 months of age, the mice in the control group had plaques covering 2 to 6 percent of their brains. In con-

30 trast, the immunized mice had virtually no plaques.

In a second experiment, the researchers began the injections at 11 months, when some plaques had already developed. Over a 7-month period, the control transgenic mice had a 17-fold increase in the amount of plaque in their brains, whereas those

5   who received the vaccine had a 99-percent decrease compared to the 18-month-old control transgenic mice. In some mice, some of the pre-existing plaque deposits appeared to have been removed by the treatment. It was also found that other plaque-associated damage, such as inflammation and abnormal

10   nerve cell processes, lessened as a result of the immunization.

The above is thus a preliminary study in mice and for example, scientists need to find out whether vaccinated mice remain healthy in other respects and whether memory of those vacci-

15   nated remains normal. Furthermore, because the mouse model is not a complete representation of AD (the animals do not develop neurofibrillary tangles nor do many of their neurons die), additional studies will be necessary to determine whether humans have a similar or different reaction from mice.

20   Another issue to consider is that the method may perhaps "cure" amyloid deposition but fail to stop development of dementia.

Technical issues present major challenges as well. For example it is unlikely that it is even possible, using this techno-

25   logy, to create a vaccine which enables humans to raise antibodies against their own proteins. So numerous issues of safety and effectiveness will need to be resolved before any tests in humans can be considered.

The work by Schenk et al. thus shows that if it was possible

30   to generate a strong immune response towards self-proteins in

proteinaceous deposits in the central nervus system such as
the plaques formed in AD, it is possible to both prevent the
formation of the deposits and possibly also clear already
formed plaques.

5 Recently, clinical trials using the above-discussed Aβ vaccines
have been terminated due to adverse effects: A number of the
vaccinated subjects developed chronic encephalitis that may be
due to an uncontrolled autoimmunity against Aβ in the CNS.

OBJECT OF THE INVENTION

10 The object of the present invention is to provide novel thera-
pies against conditions characterized by deposition of amy-
loid, such as AD. A further object is to develop an autovac-
cine against amyloid, in order to obtain a novel treatment for
AD and for other pathological disorders involving amyloid
15 deposition.

SUMMARY OF THE INVENTION

Described herein is the use of an autovaccination technology
for generating strong immune responses against otherwise non-
20 immunogenic APP and Aβ Described is also the preparation of
such vaccines for the prevention, possible cure or alleviation
of the symptoms of such diseases associated with amyloid de-
posits.

Thus, in its broadest and most general scope, the present in-
25 vention relates to a method for *in vivo* down-regulation of

amyloid precursor protein (APP) or beta amyloid (Aβ) in an animal, including a human being, the method comprising effecting presentation to the animal's immune system of an immunogenically effective amount of at least one analogue of APP or Aβ

5   that incorporates into the same molecule at least one B-cell epitope of APP and/or Aβ and at least one foreign T-helper epitope (T_H epitope) so that immunization of the animal with the analogue induces production of antibodies against the animal's autologous APP or Aβ, wherein the analogue

10   a) is a polyamino acid that consists of at least one copy
        of a subsequence of residues 672-714 in SEQ ID NO: 2,
        wherein the foreign T_H epitope is incorporated by means
        of amino acid addition and/or insertion and/or deletion
        and/or substitution, wherein the subsequence is se-
15   lected from the group consisting of residues 1-42,
        residues 1-40, residues 1-39, residues 1-35, residues
        1-34, residues 1-28, residues 1-12, residues 1-5, resi-
        dues 13-28, residues 13-35, residues 17-28, residues
        25-35, residues 35-40, residues 36-42 and residues 35-
20   42 of the amino acid sequence consisting of amino acid
        residues 673-714 of SEQ ID NO: 2; and/or

    b) is a polyamino acid that contains the foreign T_H epi-
        topes and a disrupted APP or Aβ sequence so that the
        analogue does not include any subsequence of SEQ ID NO:
25   2 that binds productively to MHC class II molecules
        initiating a T-cell response; and/or

    c) is a polyamino acid that comprises the foreign T_H epi-
        tope and APP or Aβ derived amino acids, and comprises 1
        single methionine residue located in the C-terminus of
30   the analogue, wherein other methionine residues in APP

or Aβ and in the foreign T_H epitope have been substituted or deleted, and preferably have been substituted by leucin or isoleucine; and/or

d) is a conjugate comprising a polyhydroxypolymer backbone to which is separately coupled a polyamino acid as defined in a) and/or a polyamino acid as defined in b) and/or a polyamino acid as defined in c); and/or

e) is a conjugate comprising a polyhydroxypolymer backbone to which is separately coupled 1) the foreign T_H epitope and 2) a polyamino acid selected from the group consisting of a subsequence as defined in a), a disrupted sequence of APP or Aβ as defined in b), and an APP or Aβ derived amino acid sequence that comprises 1 single methionine residue located in the C-terminus, wherein other methionine residues in APP or Aβ and in the foreign T_H epitope have been substituted or deleted, and preferably have been substituted by leucin or isoleucine.

The present assignee has previously filed an international patent application directed to safe vaccination strategies against amyloidogenic polypeptides such as APP and Aβ, cf. WO 01/62284. This application was not published on the filing date of the present application and further does not contain details concerning the above-mentioned useful analogues of APP and Aβ.

The invention also relates to analogues of the APP and Aβ as well as to nucleic acid fragments encoding a subset of these. Also immunogenic compositions comprising the analogues or the nucleic acid fragments are part of the invention.

LEGEND TO THE FIGURE

Fig. 1: Schematic depiction of Autovac variants derived from
the amyloid precursor protein with the purpose of generating
antibody responses against the Aβ protein Aβ-43 (or C-100). The
5   APP is shown schematically at the top of the figure and the
remaining schematic constructs show that the model epitopes P2
and P30 are substituted or inserted into various truncations
of APP. In the figure, the black pattern indicates the APP
signal sequence, two-way cross-hatching is the extracellular
10 part of APP, dark vertical hatching is the transmembrane do-
main of APP, light vertical hatching is the intracellular do-
main of APP, coarse cross-hatching indicates the P30 epitope,
and fine cross-hatching indicates the P2 epitope. The full
line box indicates Aβ-42/43 and the full-line box and the dot-
15 ted line box together indicate C-100. "Abeta" denotes Aβ.

Fig. 2: Schematic depiction of an embodiment of the synthesis
of generally applicable immunogenic conjugates. Peptide A (any
antigenic sequence, e.g. an Aβ sequence described herein) and
peptide B (an amino acid sequence including a foreign T-helper
20 epitope are synthesized and mixed. After that they are con-
tacted with a suitable activated polyhydroxypolymer, peptides
A and B are attached via the activation group in a ration cor-
responding to the initial ratio between these two substances
in the peptide mixture. Cf. Example 4 for details.

25 DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present
specification and claims will be defined and explained in de-

tail in order to clarify the metes and bounds of the inven-
tion.

The terms "amyloid" and "amyloid protein" which are used
interchangeably herein denote a class of proteinaceous un-
5   branched fibrils of indeterminate length. Amyloid fibrils dis-
play characteristic staining with Congo Red and share a cross-β
structure in which the polypeptide chain is organized in
β-sheets. Amyloid is generally derived from amyloidogenic pro-
teins which have very different precursor structures but which
10 can all undergo a structural conversion to a misfolded form
that is the building block of the β-sheet helix protofilament.
Normally, the diameter of amyloid fibrils varies between about
70 to about 120 Å.

The term "amyloidogenic protein" is intended to denote a poly-
15 peptide which is involved in the formation of amyloid depo-
sits, either by being part of the deposits as such or by being
part of the biosynthetic pathway leading to the formation of
the deposits. Hence, examples of amyloidogenic proteins are
APP and Aβ, but also proteins involved in the metabolism of
20 these may be amyloidogenic proteins.

An "amyloid polypeptide" is herein intended to denote polypep-
tides comprising the amino acid sequence of the above-dis-
cussed amyloidogenic proteins derived from humans or other
mammals (or truncates thereof sharing a substantial amount of
25 B-cell epitopes with an intact amyloidogenic protein) – an
amyloidogenic polypeptide can therefore e.g. comprise substan-
tial parts of a precursor for the amyloidogenic polypeptide
(in the case of Aβ, one possible amyloid polypeptide could be
APP derived). Also unglycosylated forms of amyloidogenic poly-
30 peptides which are prepared in prokaryotic system are included

within the boundaries of the term as are forms having varying
glycosylation patterns due to the use of e.g. yeasts or other
non-mammalian eukaryotic expression systems. It should, how-
ever, be noted that when using the term "an amyloidogenic
5   polypeptide" it is intended that the polypeptide in question
is normally non-immunogenic when presented to the animal to be
treated. In other words, the amyloidogenic polypeptide is a
self-protein or is an analogue of such a self-protein which
will not normally give rise to an immune response against the
10 amyloidogenic of the animal in question.

An "analogue" is an APP or Aβ derived molecule that incorpo-
rates one or several changes in its molecular structure. Such
a change can e.g. be in the form of fusion of APP or Aβ poly-
amino acids to a suitable fusion partner (i.e. a change in
15 primary structure exclusively involving C- and/or N-terminal
additions of amino acid residues) and/or it can be in the form
of insertions and/or deletions and/or substitutions in the
polypeptide's amino acid sequence. Also encompassed by the
term are derivatized APP or Aβ derived molecules, cf. the dis-
20 cussion below of modifications of APP or Aβ. In some cases the
analogue may be constructed so as to be less able or even un-
able to elicit antibodies against the normal precursor pro-
tein(s) of the amyloid, thereby avoiding undesired interfer-
ence with the (physiologically normal) non-aggregated form of
25 the polypeptide being a precursor of the amyloid protein.

It should be noted that the use as a vaccine in a human of a
xeno-analogue (e.g. a canine or porcine analogue) of a human
APP or Aβcan be imagined to produce the desired immunity
against the APP or Aβ. Such use of an xeno-analogue for immuni-
30 zation is also considered part of the invention.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, oligopeptides of from 11 to 100 amino acid residues, and poly-peptides of more than 100 amino acid residues. Furthermore,

5   the term is also intended to include proteins, *i.e.* functional biomolecules comprising at least one polypeptide; when compri-sing at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The poly-peptide(s) in a protein can be glycosylated and/or lipidated

10 and/or comprise prosthetic groups. Also, the term "polyamino acid" is an equivalent to the term "polypeptide"

The terms "T-lymphocyte" and "T-cell" will be used inter-changeably for lymphocytes of thymic origin which are respon-sible for various cell mediated immune responses as well as

15 for helper activity in the humoral immune response. Likewise, the terms "B-lymphocyte" and "B-cell" will be used inter-changeably for antibody-producing lymphocytes.

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleo-

20 tides, derived directly from a naturally occurring amyloid amino acid sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general in-tended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one

25 single animal. However, the term also denotes a population of such an animal species, since it is important that the indi-viduals immunized according to the method of the invention all harbour substantially the same APP or Aβ allowing for immuniza-tion of the animals with the same immunogen(s). It will be

30 clear to the skilled person that an animal in the present con-

text is a living being which has an immune system. It is pre-
ferred that the animal is a vertebrate, such as a mammal.

By the term "*in vivo* down-regulation of APP or Aβ" is herein
meant reduction in the living organism of the total amount of
5   deposited amyloid protein (or amyloid as such) of the relevant
type. The down-regulation can be obtained by means of several
mechanisms: Of these, simple interference with amyloid by an-
tibody binding so as to prevent misaggregation is the most
simple. However, it is also within the scope of the present
10 invention that the antibody binding results in removal of amy-
loid by scavenger cells (such as macrophages and other phago-
cytic cells) and that the antibodies interfer with other amy-
loidogenic polypeptides which lead to amyloid formation. A
further possibility is that antibodies bind Aβ outside the CNS,
15 thereby effectively removing Aβ from the CNS via a simple mass
action principle.

The expression "effecting presentation ... to the immune sys-
tem" is intended to denote that the animal's immune system is
subjected to an immunogenic challenge in a controlled manner.
20 As will appear from the disclosure below, such challenge of
the immune system can be effected in a number of ways of which
the most important are vaccination with polypeptide containing
"pharmaccines" (*i.e.* a vaccine which is administered to treat
or ameliorate ongoing disease) or nucleic acid "pharmaccine"
25 vaccination. The important result to achieve is that immune
competent cells in the animal are confronted with the antigen
in an immunologically effective manner, whereas the precise
mode of achieving this result is of less importance to the in-
ventive idea underlying the present invention.

23

The term "immunogenically effective amount" has its usual
meaning in the art, *i.e.* an amount of an immunogen, which is
capable of inducing an immune response that significantly en-
gages pathogenic agents sharing immunological features with
5 the immunogen.

When using the expression that the APP or Aβ has been "modi-
fied" is herein meant that a chemical modification of the
polypeptide has been performed on APP or Aβ. Such a modifica-
tion can e.g. be derivatization (e.g. alkylation) of certain
10 amino acid residues in the sequence, but as will be appreci-
ated from the disclosure below, the preferred modifications
comprise changes of the primary structure of the amino acid
sequence.

When discussing "autotolerance towards APP or Aβ" it is under-
15 stood that since the polypeptide is a self-protein in the
population to be vaccinated, normal individuals in the popula-
tion do not mount an immune response against the polypeptide;
it cannot be excluded, though, that occasional individuals in
an animal population might be able to produce antibodies
20 against the native polypeptide, e.g. as part of an autoimmune
disorder. At any rate, an animal will normally only be
autotolerant towards its own APP or Aβ, but it cannot be ex-
cluded that analogues derived from other animal species or
from a population having a different phenotype would also be
25 tolerated by said animal.

A "foreign T-cell epitope" (or: "foreign T-lymphocyte epi-
tope") is a peptide which is able to bind to an MHC molecule
and which stimulates T-cells in an animal species. Preferred
foreign T-cell epitopes in the invention are "promiscuous"
30 epitopes, *i.e.* epitopes which bind to a substantial fraction

24

of a particular class of MHC molecules in an animal species or
population. Only a very limited number of such promiscuous T-
cell epitopes are known, and they will be discussed in detail
below. Promiscuous T-cell epitopes are also denoted "univer-
5   sal" T-cell epitopes. It should be noted that in order for the
immunogens which are used according to the present invention
to be effective in as large a fraction of an animal population
as possible, it may be necessary to 1) insert several foreign
T-cell epitopes in the same analogue or 2) prepare several
10 analogues wherein each analogue has a different promiscuous
epitope inserted. It should be noted also that the concept of
foreign T-cell epitopes also encompasses use of cryptic T-cell
epitopes, *i.e.* epitopes which are derived from a self-protein
and which only exerts immunogenic behaviour when existing in
15 isolated form without being part of the self-protein in ques-
tion.

A "foreign T helper lymphocyte epitope" (a foreign T_H epitope)
is a foreign T cell epitope, which binds an MHC Class II mole-
cule and can be presented on the surface of an antigen pre-
20 senting cell (APC) bound to the MHC Class II molecule.

A "functional part" of a (bio)molecule is in the present con-
text intended to mean the part of the molecule which is re-
sponsible for at least one of the biochemical or physiological
effects exerted by the molecule. It is well-known in the art
25 that many enzymes and other effector molecules have an active
site which is responsible for the effects exerted by the mole-
cule in question. Other parts of the molecule may serve a sta-
bilizing or solubility enhancing purpose and can therefore be
left out if these purposes are not of relevance in the context
30 of a certain embodiment of the present invention. For instance
it is possible to use certain cytokines as a modifying moiety

in APP or Aβ (cf. the detailed discussion below), and in such a case, the issue of stability may be irrelevant since the coupling to the APP or Aβmay provide the stability necessary.

The term "adjuvant" has its usual meaning in the art of vaccine technology, *i.e.* a substance or a composition of matter which is 1) not in itself capable of mounting a specific immune response against the immunogen of the vaccine, but which is 2) nevertheless capable of enhancing the immune response against the immunogen. Or, in other words, vaccination with the adjuvant alone does not provide an immune response against the immunogen, vaccination with the immunogen may or may not give rise to an immune response against the immunogen, but the combined vaccination with immunogen and adjuvant induces an immune response against the immunogen which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended to denote the situation where a molecule upon introduction in the animal will appear preferentially in certain tissue(s) or will be preferentially associated with certain cells or cell types. The effect can be accomplished in a number of ways including formulation of the molecule in composition facilitating targeting or by introduction in the molecule of groups, which facilitate targeting. These issues will be discussed in detail below.

"Stimulation of the immune system" means that a substance or composition of matter exhibits a general, non-specific immunostimulatory effect. A number of adjuvants and putative adjuvants (such as certain cytokines) share the ability to stimulate the immune system. The result of using an immunostimulating agent is an increased "alertness" of the immune system

meaning that simultaneous or subsequent immunization with an immunogen induces a significantly more effective immune response compared to isolated use of the immunogen.

"Productive binding" means binding of a peptide to the MHC

5 molecule (Class I or II) so as to be able to stimulate T-cells that engage a cell that present the peptide bound to the MHC molecule. For instance, a peptide bound to an MHC Class II molecule on the surface of an APC is said to be productively bound if this APC will stimulate a T_H cell that binds to the

10 presented peptide-MHC Class II complex.

Preferred embodiments of amyloid down-regulation

It is preferred that the analogue used as an immunogen in the method of the invention is a modified APP or Aβ molecule wherein at least one change is present in the amino acid se-

15 quence of the APP or Aβ, since the chances of obtaining the all-important breaking of autotolerance is greatly facilitated that way – this is e.g. evident from the results presented in Example 2 herein, where immunization with wild-type Aβ is compared to immunization with an Aβ variant molecule. It has been

20 shown (in Dalum I et al., 1996, J. Immunol. **157**: 4796-4804) that potentially self-reactive B-lymphocytes recognizing self-proteins are physiologically present in normal individuals. However, in order for these B-lymphocytes to be induced to actually produce antibodies reactive with the relevant self-pro-

25 teins, assistance is needed from cytokine producing T-helper lymphocytes (T_H-cells or T_H-lymphocytes). Normally this help is not provided because T-lymphocytes in general do not recognize T-cell epitopes derived from self-proteins when presented by antigen presenting cells (APCs). However, by providing an ele-

30 ment of "foreignness" in a self-protein (i.e. by introducing

an immunologically significant modification), T-cells recog-
nizing the foreign element are activated upon recognizing the
foreign epitope on an APC (such as, initially, a mononuclear
cell). Polyclonal B-lymphocytes (which are also APCs) capable

5 of recognising self-epitopes on the modified self-protein also
internalise the antigen and subsequently presents the foreign
T-cell epitope(s) thereof, and the activated T-lymphocytes
subsequently provide cytokine help to these self-reactive
polyclonal B-lymphocytes. Since the antibodies produced by

10 these polyclonal B-lymphocytes are reactive with different
epitopes on the modified polypeptide, including those which
are also present in the native polypeptide, an antibody cross-
reactive with the non-modified self-protein is induced. In
conclusion, the T-lymphocytes can be led to act as if the

15 population of polyclonal B-lymphocytes have recognised an en-
tirely foreign antigen, whereas in fact only the inserted epi-
tope(s) is/are foreign to the host. In this way, antibodies
capable of *cross-reacting* with non-modified self-antigens are
induced.

20 Several ways of modifying a peptide self-antigen in order to
obtain breaking of autotolerance are known in the art. It is
nevertheless preferred that the analogue according to the pre-
sent invention includes

-       at least one first moiety is introduced which effects
25   targeting of the modified molecule to an antigen presen-
        ting cell (APC), and/or

-       at least one second moiety is introduced which stimulates
        the immune system, and/or

-       at least one third moiety is introduced which optimizes
30   presentation of the analogue to the immune system.

However, all these modifications should be carried out while maintaining a substantial fraction of the original B-lymphocyte epitopes in the APP or Aβ, since the B-lymphocyte recognition of the native molecule is thereby enhanced.

5   In one preferred embodiment, side groups (in the form of the foreign T-cell epitopes or the above-mentioned first, second and third moieties) are covalently or non-covalently introduced. This is to mean that stretches of amino acid residues derived from the APP or Aβ are derivatized without altering the

10   primary amino acid sequence, or at least without introducing changes in the peptide bonds between the individual amino acids in the chain.

An alternative, and preferred, embodiment utilises amino acid substitution and/or deletion and/or insertion and/or addition

15   (which may be effected by recombinant means or by means of peptide synthesis; modifications which involves longer stretches of amino acids can give rise to fusion polypeptides). One especially preferred version of this embodiment is the technique described in WO 95/05849, which discloses a

20   method for down-regulating self-proteins by immunising with analogues of the self-proteins wherein a number of amino acid sequence(s) has been substituted with a corresponding number of amino acid sequence(s) which each comprise a foreign immunodominant T-cell epitope, while at the same time maintaining

25   the overall tertiary structure of the self-protein in the analogue. For the purposes of the present invention, it is however sufficient if the modification (be it an insertion, addition, deletion or substitution) gives rise to a foreign T-cell epitope and at the same time preserves a substantial number of

30   the B-cell epitopes in the APP or Aβ. However, in order to obtain maximum efficacy of the immune response induced, it is

29

preferred that the overall tertiary structure of the APP or Aβ
is maintained in the modified molecule.

In some cases, it is preferred that the APP or Aβ or fragments
thereof are mutated. Especially preferred are substitution

5 variants where the methionine in position 35 in Aβ-43 has been
substituted, preferably with leucine or isoleucine, or simply
deleted. Especially preferred analogues contain one single me-
thionine that is located in the C-terminus, either because it
is naturally occurring in the amyloidogenic polypeptide or

10 foreign T$_H$ epitope, or because it has been inserted or added.
Hence, it also preferred that the part of the analogue that
includes the foreign T$_H$ epitope is free from methionine, except
from the possible C-terminal location of a methionine.

The main reason for removing all but one methionine is that it

15 becomes possible to recombinantly prepare multimeric analogues
that can be subsequently cleaved by cyanogenbromide to leave
the single analogues. The advantage is, that recombinant
production becomes facilitated this way.

In fact, it is generally preferred that all analogues of APP

20 or Aβ that are used according to the present invention share
the characteristic of merely including one single methionine
that is positioned as the C-terminal amino acid in the
analogue and that other methionines in either the
amyloidogenic polypeptide or the foreign T$_H$ epitope are deleted

25 or substituted for another amino acid.

One further interesting mutation is a deletion or substitution
of the phenylalanine in position 19 in Aβ-43, and it is espe-
cially preferred that the mutation is a substitution of this
phenylalanine residue with a proline.

Other interesting polyamino acids to be used in the analogues
are truncated parts of the Aβ-43 protein. These can also be
employed in immunogenic analogues according to the present
invention. Especially preferred are the truncates Aβ(1-42),
5   Aβ(1-40), Aβ(1-39), Aβ(1-35), Aβ(1-34), Aβ(1-34), Aβ(1-28),
Aβ(1-12), Aβ(1-5), Aβ(13-28), Aβ(13-35), Aβ(17-28), Aβ(25-35),
Aβ(35-40), Aβ(36-42), and Aβ(35-42) (where the numbers in the
parentheses indicate the amino acid stretches of Aβ-43 that
constitute the relevant fragment – Aβ(35-40) is e.g. identical
10   to amino acids 706-711 in SEQ ID NO: 2). All these variants
with truncated parts of Aβ-43 can be made with the Aβ fragments
described herein, in particular with variants 9, 10, 11, 12,
and 13 mentioned in Example 1.

The following formula describes the molecular constructs gene-
15   rally covered by the invention:

$$(MOD_1)_{s1}(amyloid_{e1})_{n1}(MOD_2)_{s2}(amyloid_{e2})_{n2}\ldots(MOD_x)_{sx}(amyloid_{ex})_{nx} \quad (I)$$
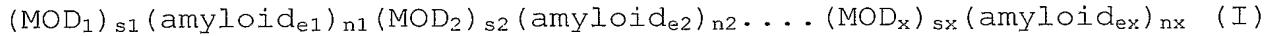
-where $amyloid_{e1}$-$amyloid_{ex}$ are x B-cell epitope containing sub-
sequences of APP or Aβ which independently are identical or
non-identical and which may contain or not contain foreign
20   side groups, x is an integer ≥ 3, n1-nx are x integers ≥ 0 (at
least one is ≥ 1), MOD_1-MOD_x are x modifications introduced be-
tween the preserved B-cell epitopes, and s_1-s_x are x integers ≥
0 (at least one is ≥ 1 if no side groups are introduced in the
$amyloid_{ex}$ sequences). Thus, given the general functional re-
25   straints on the immunogenicity of the constructs, the inven-
tion allows for all kinds of permutations of the original se-
quence of the APP or Aβ, and all kinds of modifications
therein. Thus, included in the invention are modified APP or Aβ
obtained by omission of parts of the sequence which e.g. ex-

hibit adverse effects *in vivo* or omission of parts which are normally intracellular and thus could give rise to undesired immunological reactions.

One preferred version of the constructs outlined above are, when applicable, those where the B-cell epitope containing subsequence of an amyloid protein is not extracellularly exposed in the precursor polypeptide from which the amyloid is derived. By making such a choice of the epitopes, it is ensured that antibodies are not generated which would be reactive with the cells producing the precursor and thereby the immune response which is generated becomes limited to an immune response against the undesired amyloid deposits. In this case it will e.g. be feasible to induce immunity against epitopes of APP or Aβ which are only exposed to the extracellular phase when being free from any coupling to the cells from which they are produced.

Maintenance of a substantial fraction of B-cell epitopes or even the overall tertiary structure of a protein which is subjected to modification as described herein can be achieved in several ways. One is simply to prepare a polyclonal antiserum directed against the polypeptide in question (e.g. an antiserum prepared in a rabbit) and thereafter use this antiserum as a test reagent (e.g. in a competitive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does the APP or Aβ must be regarded as having the same overall tertiary structure as APP or Aβ whereas analogues exhibiting a limited (but still significant and specific) reactivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive with distinct epitopes on the APP or Aβ can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of the APP or Aβ and 2) a mapping
5 of the epitopes which are maintained in the analogues prepared.

Of course, a third approach would be to resolve the 3-dimensional structure of the APP or Aβ or of a biologically active truncate thereof (cf. above) and compare this to the resolved
10 three-dimensional structure of the analogues prepared. Three-dimensional structure can be resolved by the aid of X-ray diffraction studies and NMR-spectroscopy. Further information relating to the tertiary structure can to some extent be obtained from circular dichroism studies which have the advan-
15 tage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide and NMR requires the provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule. However, ulti-
20 mately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism can only provide indirect evidence of correct 3-dimensional structure via information of secondary structure elements.

One preferred embodiment of the invention utilises multiple
25 presentations of B-lymphocyte epitopes of APP or Aβ (*i.e.* formula I wherein at least one B-cell epitope is present in two positions). This effect can be achieved in various ways, e.g. by simply preparing fusion polypeptides comprising the structure (APP or Aβ derived polypeptide)$_m$, where m is an integer $\geq$
30 2 and then introduce the modifications discussed herein in at

least one of the APP or Aβ sequences. It is preferred that the
modifications introduced includes at least one duplication of
a B-lymphocyte epitope and/or the introduction of a hapten.
These embodiments including multiple presentations of selected
5 epitopes are especially preferred in situations where merely
minor parts of the APP or Aβ are useful as constituents in a
vaccine agent.

As mentioned above, the introduction of a foreign T-cell epi-
tope can be accomplished by introduction of at least one amino
10 acid insertion, addition, deletion, or substitution. Of
course, the normal situation will be the introduction of more
than one change in the amino acid sequence (e.g. insertion of
or substitution by a complete T-cell epitope) but the impor-
tant goal to reach is that the analogue, when processed by an
15 antigen presenting cell (APC), will give rise to such a for-
eign immunodominant T-cell epitope being presented in context
of an MCH Class II molecule on the surface of the APC. Thus,
if the amino acid sequence of the APP or Aβ in appropriate po-
sitions comprises a number of amino acid residues which can
20 also be found in a foreign T_H epitope then the introduction of
a foreign T_H epitope can be accomplished by providing the re-
maining amino acids of the foreign epitope by means of amino
acid insertion, addition, deletion and substitution. In other
words, it is not necessary to introduce a complete T_H epitope
25 by insertion or substitution in order to fulfill the purpose
of the present invention.

It is preferred that the number of amino acid insertions, de-
letions, substitutions or additions is at least 2, such as 3,
4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20,
30 and 25 insertions, substitutions, additions or deletions. It
is furthermore preferred that the number of amino acid inser-

34

tions, substitutions, additions or deletions is not in excess
of 150, such as at most 100, at most 90, at most 80, and at
most 70. It is especially preferred that the number of substi-
tutions, insertions, deletions, or additions does not exceed

5   60, and in particular the number should not exceed 50 or even
40. Most preferred is a number of not more than 30. With re-
spect to amino acid additions, it should be noted that these,
when the resulting construct is in the form of a fusion poly-
peptide, is often considerably higher than 150.

10   Preferred embodiments of the invention includes modification
by introducing at least one foreign immunodominant T-cell epi-
tope. It will be understood that the question of immune domi-
nance of a T-cell epitope depends on the animal species in
question. As used herein, the term "immunodominance" simply

15   refers to epitopes which in the vaccinated indivi-
dual/population gives rise to a significant immune response,
but it is a well-known fact that a T-cell epitope which is im-
munodominant in one individual/population is not necessarily
immunodominant in another individual of the same species, even

20   though it may be capable of binding MHC-II molecules in the
latter individual. Hence, for the purposes of the present in-
vention, an immune dominant T-cell epitope is a T-cell epitope
which will be effective in providing T-cell help when present
in an antigen. Typically, immune dominant T-cell epitopes has

25   as an inherent feature that they will substantially always be
presented bound to an MHC Class II molecule, irrespective of
the polypeptide wherein they appear.

Another important point is the issue of MHC restriction of T-
cell epitopes. In general, naturally occurring T-cell epitopes

30   are MHC restricted, *i.e.* a certain peptides constituting a T-
cell epitope will only bind effectively to a subset of MHC

35

Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are variants of APP or Aβ which are distinguished from each other by the nature of the T-cell epitope introduced.

If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be approximated by means of the following formula

$$f_{population} = 1 - \prod_{i=1}^{n}(1 - p_i) \tag{II}$$

-where p_i is the frequency in the population of responders to the ith foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-i.e. 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and

36

DR7, then the use of this peptide together with another pep-
tide which binds the remaining MHC-II molecules encoded by
HLA-DR alleles will accomplish 100% coverage in the population
in question. Likewise, if the second peptide only binds DR3

5   and DR5, the addition of this peptide will not increase the
coverage at all. If one bases the calculation of population
response purely on MHC restriction of T-cell epitopes in the
vaccine, the minimum fraction of the population covered by a
specific vaccine composition can be determined by means of the

10   following formula:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 \qquad (III)$$

-wherein φ_j is the sum of frequencies in the population of al-
lelic haplotypes encoding MHC molecules which bind any one of
the T-cell epitopes in the vaccine and which belong to the jth

15   of the 3 known HLA loci (DP, DR and DQ); in practice, it is
first determined which MHC molecules will recognize each T-
cell epitope in the vaccine and thereafter these are listed by
type (DP, DR and DQ) – then, the individual frequencies of the
different listed allelic haplotypes are summed for each type,

20   thereby yielding φ_1, φ_2, and φ_3.

It may occur that the value p_i in formula II exceeds the corre-
sponding theoretical value π_i:

$$\pi_i = 1 - \prod_{j=1}^{3} (1 - v_j)^2 \qquad (IV)$$

-wherein v_j is the sum of frequencies in the population of al-

25   lelic haplotype encoding MHC molecules which bind the ith T-
cell epitope in the vaccine and which belong to the jth of the
3 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of
the population is a frequency of responders of $f_{residual\_i} = (p_i-$

37

n_i)/(1−n_i). Therefore, formula III can be adjusted so as to yield formula V:

$$f_{population} = 1 - \prod_{j=1}^{3}(1-\varphi_j)^2 + \left(1 - \prod_{i=1}^{n}(1-f_{residual\_i})\right) \qquad (V)$$

—where the term 1−$f_{residual-i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine thereby reducing the need for a very large number of different analogues in the same vaccine.

The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagluttinin (HA), and *P. falciparum* CS antigen.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible T-cell epitopes to be introduced in the analogues used according to the present invention. Cf. also the epitopes discussed

in the following references which are hereby all incorporated
by reference herein: WO 98/23635 (Frazer IH *et al.*, assigned
to The University of Queensland); Southwood S *et. al*, 1998, J.
Immunol. **160**: 3363-3373; Sinigaglia F *et al.*, 1988, Nature
5   **336**: 778-780; Chicz RM *et al.*, 1993, J. Exp. Med **178**: 27-47;
Hammer J *et al.*, 1993, Cell **74**: 197-203; and Falk K *et al.*,
1994, Immunogenetics **39**: 230-242. The latter reference also
deals with HLA-DQ and -DP ligands. All epitopes listed in
these 5 references are relevant as candidate natural epitopes
10 to be used in the present invention, as are epitopes which
share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epi-
tope which is capable of binding a large proportion of MHC
Class II molecules. In this context the pan DR epitope pep-
15 tides ("PADRE") described in WO 95/07707 and in the corre-
sponding paper Alexander J *et al.*, 1994, Immunity **1**: 751-761
(both disclosures are incorporated by reference herein) are
interesting candidates for epitopes to be used according to
the present invention. It should be noted that the most effec-
20 tive PADRE peptides disclosed in these papers carry D-amino
acids in the C- and N-termini in order to improve stability
when administered. However, the present invention primarily
aims at incorporating the relevant epitopes as part of the
analogue which should then subsequently be broken down enzy-
25 matically inside the lysosomal compartment of APCs to allow
subsequent presentation in the context of an MHC-II molecule
and therefore it is not expedient to incorporate D-amino acids
in the epitopes used in the present invention.

One especially preferred PADRE peptide is the one having the
30 amino acid sequence AKFVAAWTLKAAA (SEQ ID NO: 17) or an immu-
nologically effective subsequence thereof. This, and other

epitopes having the same lack of MHC restriction are preferred
T-cell epitopes which should be present in the analogues used
in the inventive method. Such super-promiscuous epitopes will
allow for the most simple embodiments of the invention wherein
5   only one single analogue is presented to the vaccinated ani-
mal's immune system.

As mentioned above, the modification of the APP or Aβ can also
include the introduction of a first moiety which targets the
modified amyloidogenic polypeptide to an APC or a B-lympho-
10 cyte. For instance, the first moiety can be a specific binding
partner for a B-lymphocyte specific surface antigen or for an
APC specific surface antigen. Many such specific surface anti-
gens are known in the art. For instance, the moiety can be a
carbohydrate for which there is a receptor on the B-lymphocyte
15 or the APC (e.g. mannan or mannose). Alternatively, the second
moiety can be a hapten. Also an antibody fragment which spe-
cifically recognizes a surface molecule on APCs or lymphocytes
can be used as a first moiety (the surface molecule can e.g.
be an FCγ receptor of macrophages and monocytes, such as FCγRI
20 or, alternatively any other specific surface marker such as
CD40 or CTLA-4). It should be noted that all these exemplary
targeting molecules can be used as part of an adjuvant also,
cf. below.

As an alternative or supplement to targeting the analogue to a
25 certain cell type in order to achieve an enhanced immune re-
sponse, it is possible to increase the level of responsiveness
of the immune system by including the above-mentioned second
moiety which stimulates the immune system. Typical examples of
such second moieties are cytokines, and heat-shock proteins or
30 molecular chaperones, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are those which will normally also function as adjuvants in a vaccine composition, *i.e.* for instance interferon γ (IFN-γ), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-

5 4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF); alternatively, the functional part of the cytokine molecule may suffice as the second moiety. With respect to the use of such cytokines as adjuvant

10 substances, cf. the discussion below.

According to the invention, suitable heat-shock proteins or molecular chaperones used as the second moiety can be HSP70, HSP90, HSC70, GRP94 (also known as gp96, cf. Wearsch PA *et al.* 1998, Biochemistry **37**: 5709-19), and CRT (calreticulin).

15 Alternatively, the second moiety can be a toxin, such as listeriolycin (LLO), lipid A and heat-labile enterotoxin. Also, a number of mycobacterial derivatives such as MDP (muramyl dipeptide), CFA (complete Freund's adjuvant) and the trehalose diesters TDM and TDE are interesting possibilities.

20 Also the possibility of introducing a third moiety which enhances the presentation of the analogue to the immune system is an important embodiment of the invention. The art has shown several examples of this principle. For instance, it is known that the palmitoyl lipidation anchor in the *Borrelia burgdor-*

25 *feri* protein OspA can be utilised so as to provide self-adjuvating polypeptides (cf. e.g. WO 96/40718) - it seems that the lipidated proteins form up micelle-like structures with a core consisting of the lipidation anchor parts of the polypeptides and the remaining parts of the molecule protruding therefrom,

30 resulting in multiple presentations of the antigenic determi-

nants. Hence, the use of this and related approaches using
different lipidation anchors (e.g. a myristyl group, a myris-
tyl group, a farnesyl group, a geranyl-geranyl group, a GPI-
anchor, and an N-acyl diglyceride group) are preferred embodi-
5   ments of the invention, especially since the provision of such
a lipidation anchor in a recombinantly produced protein is
fairly straightforward and merely requires use of e.g. a natu-
rally occurring signal sequence as a fusion partner for the
analogue. Another possibility is use of the C3d fragment of
10 complement factor C3 or C3 itself (cf. Dempsey *et al.*, 1996,
Science **271**, 348-350 and Lou & Kohler, 1998, Nature Biotech-
nology **16**, 458-462).

An alternative embodiment of the invention which also results
in the preferred presentation of multiple (e.g. at least 2)
15 copies of the important epitopic regions of APP or Aβ to the
immune system is the covalent coupling of the analogue to cer-
tain molecules, *i.e.* variants d and e mentioned above. For
instance, polymers can be used, e.g. carbohydrates such as
dextran, cf. e.g. Lees A et al., 1994, Vaccine 12: 1160-1166;
20 Lees A et al., 1990, J Immunol. 145: 3594-3600, but also
mannose and mannan are useful alternatives. Integral membrane
proteins from e.g. E. coli and other bacteria are also useful
conjugation partners. The traditional carrier molecules such
as keyhole limpet hemocyanin (KLH), tetanus toxoid, diphtheria
25 toxoid, and bovine serum albumin (BSA) are also preferred and
useful conjugation partners.

Preferred embodiments of covalent coupling of the APP or Aβ de-
rived material to polyhydroxypolymers such as carbohydrates
involve the use of at least one APP or Aβ derived peptide and
30 at least one foreign T-helper epitope which are coupled sepa-
rately to the polyhydroxypolymer (i.e. the foreign T-helper

epitope and the APP or Aβ derived amino acid sequence are not
fused to each other but rather bound to the polyhydroxypolymer
which then serves as a carrier backbone). Again, such an em-
bodiment is most preferred when the suitable B-cell epitope
5 carrying regions of the APP or Aβ derived peptides are consti-
tuted by short peptide stretches – this is because this ap-
proach is one very convenient way to achieve multiple presen-
tations of selected epitopes in the resulting immunogenic
agent. It is, however, also possible to simply coupled ana-
10 logues already described herein to the polyhydroxypolymer
backbone, *i.e.* that the APP or Aβ derived material is not at-
tached to the backbone separately from the foreign T$_H$ epitopes.

It is especially preferred that the coupling of the foreign T-
helper epitope and the APP or Aβ derived (poly)peptide is by
15 means of an amide bond which can be cleaved by a peptidase.
This strategy has the effect that APCs will be able to take up
the conjugate and at the same time be able to process the con-
jugate and subsequently present the foreign T-cell epitope in
an MHC Class II context.

20 One way of achieving coupling of peptides (both the APP or Aβ
derived peptide of interest as well as the foreign epitope) is
to activate a suitable polyhydroxypolymer with tresyl
(trifluoroethylsulphonyl) groups or other suitable activation
groups such as maleimido, p-Nitrophenyl cloroformate (for ac-
25 tivation of OH groups and formation of a peptide bond between
peptide and polyhydroxypolymer), and tosyl (p-toluenesul-
fonyl). It is e.g. possible to prepare activated polysaccha-
rides as described in WO 00/05316 and US 5,874,469 (both in-
corporated by reference herein) and couple these to APP or Aβ
30 derived peptides or polyamino acids as well as to T-cell epi-

topes prepared by means of conventional solid or liquid phase peptide synthesis techniques. The resulting product consists of a polyhydroxypolymer backbone (e.g. a dextran backbone) that has, attached thereto by their N-termini or by other

5 available nitrogen moieties, polyamino acids derived from APP or Aβ and from foreign T-cell epitopes. If desired, it is possible to synthesise the APP or Aβ peptides so as to protect all available amino groups but the one at the N-terminus, subsequently couple the resulting protected peptides to the tresy-

10 lated dextran moiety, and finally de-protecting the resulting conjugate. A specific example of this approach is described in the examples below.

Instead of using the water-soluble polysaccharide molecules as taught in WO 00/05316 and US 5,874,469, it is equally possible

15 to utilise cross-linked polysaccharide molecules, thereby obtaining a particulate conjugate between polypeptides and polysaccharide – this is believed to lead to an improved presentation to the immune system of the polypeptides, since two goals are reached, namely to obtain a local deposit effect when in-

20 jecting the conjugate and to obtain particles which are attractive targets for APCs. The approach of using such particulate systems is also detailed in the examples.

Considerations underlying chosen areas of introducing modifications in APP or Aβ are a) preservation of known and

25 predicted B-cell epitopes, b) preservation of tertiary structure, c) avoidance of B-cell epitopes present on "producer cells" etc. At any rate, as discussed above, it is fairly easy to screen a set of analogues which have all been subjected to introduction of a T-cell epitope in different locations.

Since the most preferred embodiments of the present invention
involve down-regulation of human Aβ, it is consequently pre-
ferred that the APP or Aβpolypeptide discussed above is a human
Aβ polypeptide. In this embodiment, it is especially preferred

5   that the APP or Aβ polypeptide has been modified by substitu-
ting at least one amino acid sequence in SEQ ID NO: 2 with at
least one amino acid sequence of equal or different length and
containing a foreign T_H epitope. Preferred examples of modified
amyloidogenic APP and Aβ are shown schematically in Fig. 1 us-

10   ing the P2 and P30 epitopes as examples. The rationale behind
such constructs is discussed in detail in the examples.

More specifically, a T_H containing (or completing) amino acid
sequence which is introduced into SEQ ID NO: 2 may be intro-
duced at any amino acid in SEQ ID NO: 2. That is, the intro-

15   duction is possible after any of amino acids 1-770, but pre-
ferably after any of amino acids 671, 672, 673, 674, 675, 676,
677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688,
689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700,
701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712,

20   713, and 714 in SEQ ID NO: 2. This may be combined with dele-
tion of any or all of amino acids 1-671, or any of all of
amino acids 715-770. Furthermore, when utilising the technique
of substitution, any one of amino acids 671, 672, 673, 674,
675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686,

25   687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698,
699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710,
711, 712, 713, and 714 in SEQ ID NO: 2 may be deleted in com-
bination with the introduction.

Another embodiment of the present invention is the presenta-

30   tion of the analogues which do not include any subsequence of

of SEQ ID NO: 2 that binds productively to MHC class II mole-
cules initiating a T-cell response.

The rationale behind such a strategy for design of the immuno-
gen that engages the immune system to induce e.g. an anti-Aβ
5   immune response is the following: It has been noted that when
immunizing with abundant autologous proteins such as Aβ formu-
lated in an adjuvant which is sufficiently strong to break the
body's tolerance towards the autologous protein, there is a
danger that in some vaccinated individuals the immune response
10 induced cannot be discontinued simply by discontinueing the
immunisation. This is because the induced immune response in
such individuals is most likely driven by a native T_H epitope
of the autologous protein, and this has the adverse effect
that the vaccinated individual's own protein will be able to
15 function as an immunizing agent in its own right: An autoim-
mune condition has thus been established.

The preferred methods including use of foreign T_H epitopes have
to the best of the inventors' knowledge never been observed to
produce this effect, because the anti-self immune response is
20 driven by a foreign T_H epitope, and it has been repeatedly dem-
onstrated by the inventors that the induced immune response
invoked by the preferred technology indeed declines after dis-
continuation of immunizations. However, in theory it could
happen in a few individuals that the immune response would
25 also be driven by an autologous T_H epitope of the relevant
self-protein one immunises against) – this is especially rele-
vant when considering self-proteins that are relatively abun-
dant, such as Aβ, whereas other therapeutically relevant self-
proteins are only present locally or in so low amounts in the
30 body, that a "self-immunization effect" is not a possibility.
One very simple way of avoiding this is hence to altogether

avoid inclusion in the immunogen of peptide sequences that
could serve as T$_H$ epitopes (and since peptides shorter than
about 9 amino acids cannot serve as T$_H$ epitopes, the use of
shorter fragments is one simple and feasible approach). There-
5 fore, this embodiment of the invention also serves to ensure
that the immunogen does not indlude peptide sequences of the
target APP or Aβ that could serve as "self-stimulating T$_H$ epi-
topes" including sequences that merely contain conservative
substitutions in a sequence of the target protein that might
10 otherwise function as a T$_H$ epitope.

Preferred embodiments of the immune system presentation of the
analogues of the APP or Aβ involve the use of a chimeric pep-
tide comprising at least one APP or Aβ derived peptide, which
does not bind productively to MHC class II molecules, and at
15 least one foreign T-helper epitope. Moreover, it is preferred
that the APP or Aβ derived peptide harbours a B-cell epitope.
It is especially advantageous if the immunogenic analogue is
one, wherein the amino acid sequences comprising one or more
B-cell epitopes are represented either as a continuous se-
20 quence or as a sequence including inserts, wherein the inserts
comprise foreign T-helper epitopes.

Again, such an embodiment is most preferred when the suitable
B-cell epitope carrying regions of the APP or Aβ are consti-
tuted by short peptide stretches that in no way would be able
25 to bind productively to an MHC Class II molecule. The selected
B-cell epitope or -epitopes of the amyloidogenic polypeptide
should therefore comprise at most 9 consecutive amino acids of
SEQ ID NO: 2. Shorter peptides are preferred, such as those
having at most 8, 7, 6, 5, 4, or 3 consecutive amino acids
30 from the amyloidogenic polypeptide's amino acid sequence.

It is preferred that the analogue comprises at least one sub-
sequence of SEQ ID NO: 2 so that each such at least one subse-
quence independently consists of amino acid stretches from the
APP or Aβ selected from the group consisting of 9 consecutive
amino acids, 8 consecutive amino acids, 7 consecutive amino
acids, 6 consecutive amino acids, 5 consecutive amino acids, 4
consecutive amino acids, and 3 consecutive amino acids.

It is especially preferred that the consecutive amino acids
begins at an amino acid residue selected from the group con-
sisting of residue 672, 673, 674, 675, 676, 677, 678, 679,
680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691,
692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703,
704, 705, 706, 707, 708, 709, 710, 711, 712, 713, and 714 of
SEQ ID NO: 2.

Protein/peptide vaccination; formulation and administration of
the analogues

When effecting presentation of the analogue to an animal's im-
mune system by means of administration thereof to the animal,
the formulation of the polypeptide follows the principles gen-
erally acknowledged in the art.

Preparation of vaccines which contain peptide sequences as ac-
tive ingredients is generally well understood in the art, as
exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231;
4,599,230; 4,596,792; and 4,578,770, all incorporated herein
by reference. Typically, such vaccines are prepared as in-
jectables either as liquid solutions or suspensions; solid
forms suitable for solution in, or suspension in, liquid prior
to injection may also be prepared. The preparation may also be
emulsified. The active immunogenic ingredient is often mixed
with excipients which are pharmaceutically acceptable and com-

48

patible with the active ingredient. Suitable excipients are,
for example, water, saline, dextrose, glycerol, ethanol, or
the like, and combinations thereof. In addition, if desired,
the vaccine may contain minor amounts of auxiliary substances
5   such as wetting or emulsifying agents, pH buffering agents, or
adjuvants which enhance the effectiveness of the vaccines; cf.
the detailed discussion of adjuvants below.

The vaccines are conventionally administered parenterally, by
injection, for example, either subcutaneously, intracutaneous-
10 ly, or intramuscularly. Additional formulations which are
suitable for other modes of administration include supposito-
ries and, in some cases, oral, buccal, sublinqual, intraperi-
toneal, intravaginal, anal, epidural, spinal, and intracranial
formulations. For suppositories, traditional binders and car-
15 riers may include, for example, polyalkalene glycols or
triglycerides; such suppositories may be formed from mixtures
containing the active ingredient in the range of 0.5% to 10%,
preferably 1-2%. Oral formulations include such normally em-
ployed excipients as, for example, pharmaceutical grades of
20 mannitol, lactose, starch, magnesium stearate, sodium saccha-
rine, cellulose, magnesium carbonate, and the like. These com-
positions take the form of solutions, suspensions, tablets,
pills, capsules, sustained release formulations or powders and
contain 10-95% of active ingredient, preferably 25-70%. For
25 oral formulations, cholera toxin is an interesting formulation
partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral
or salt forms. Pharmaceutically acceptable salts include acid
addition salts (formed with the free amino groups of the pep-
30 tide) and which are formed with inorganic acids such as, for
example, hydrochloric or phosphoric acids, or such organic a-

cids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic

5   bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be adminis-

10 tered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from

15 about 0.1 µg to 2,000 µg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 1,000 µg, preferably in the range from 1 µg to 500 µg and especially in the range from about 10 µg to 100 µg. Suitable regimens for initial administration and booster shots

20 are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are

25 applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated

30 and the formulation of the antigen.

Some of the polypeptides of the vaccine are sufficiently immu-
nogenic in a vaccine, but for some of the others the immune
response will be enhanced if the vaccine further comprises an
adjuvant substance.

5   Various methods of achieving adjuvant effect for the vaccine
are known. General principles and methods are detailed in "The
Theory and Practical Application of Adjuvants", 1995, Duncan
E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-
95170-6, and also in "Vaccines: New Generationn Immunological
10 Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press,
New York, ISBN 0-306-45283-9, both of which are hereby incor-
porated by reference herein.

It is especially preferred to use an adjuvant which can be
demonstrated to facilitate breaking of the autotolerance to
15 autoantigens; in fact, this is essential in cases where unmo-
dified amyloidogenic polypeptide is used as the active
ingredient in the autovaccine. Non-limiting examples of suit-
able adjuvants are selected from the group consisting of an
immune targeting adjuvant; an immune modulating adjuvant such
20 as a toxin, a cytokine, and a mycobacterial derivative; an oil
formulation; a polymer; a micelle forming adjuvant; a saponin;
an immunostimulating complex matrix (ISCOM matrix); a parti-
cle; DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and an
encapsulating adjuvant. In general it should be noted that the
25 disclosures above which relate to compounds and agents useful
as first, second and third moieties in the analogues also re-
fer mutatis mutandis to their use in the adjuvant of a vaccine
of the invention.

The application of adjuvants include use of agents such as
30 aluminum hydroxide or phosphate (alum), commonly used as 0.05

to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C

5 for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-

10 negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Admixture with oils such as squalene and IFA is also preferred.

15 According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant as is DNA and γ-inulin, but also Freund's complete and incomplete adjuvants as well as *quillaja* saponins such as QuilA and QS21 are interesting as is RIBI. Further possibilities are monophos-

20 phoryl lipid A (MPL), the above mentioned C3 and C3d, and muramyl dipeptide (MDP).

Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are preferred according to the invention.

25 Also immunostimulating complex matrix type (ISCOM® matrix) adjuvants are preferred choices according to the invention, especially since it has been shown that this type of adjuvants are capable of up-regulating MHC Class II expression by APCs. An ISCOM® matrix consists of (optionally fractionated) sapo-

30 nins (triterpenoids) from *Quillaja saponaria*, cholesterol, and

52

phospholipid. When admixed with the immunogenic protein, the resulting particulate formulation is what is known as an ISCOM particle where the saponin constitutes 60-70% w/w, the cholesterol and phospholipid 10-15% w/w, and the protein 10-15% w/w.

5   Details relating to composition and use of immunostimulating complexes can e.g. be found in the above-mentioned text-books dealing with adjuvants, but also Morein B et al., 1995, Clin. Immunother. **3**: 461-475 as well as Barr IG and Mitchell GF, 1996, Immunol. and Cell Biol. **74**: 8-25 (both incorporated by

10 reference herein) provide useful instructions for the preparation of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin et al., 1992 (which is hereby incorporated

15 by reference herein). In brief, the presentation of a relevant antigen such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen binding antibody fragments) against the Fcγ receptors on monocytes/macrophages. Especially conjugates between antigen and

20 anti-FcγRI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of the targeting and immune modulating substances (i.a. cytokines) mentioned above as candidates for the first and second moieties in the modified

25 versions of amyloidogenic polypeptides. In this connection, also synthetic inducers of cytokines like poly I:C are possibilities.

Suitable mycobacterial derivatives are selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant,

30 RIBI, and a diester of trehalose such as TDM and TDE.

53

Suitable immune targeting adjuvants are selected from the
group consisting of CD40 ligand and CD40 antibodies or specif-
ically binding fragments thereof (cf. the discussion above),
mannose, a Fab fragment, and CTLA-4.

5   Suitable polymer adjuvants are selected from the group con-
sisting of a carbohydrate such as dextran, PEG, starch, man-
nan, and mannose; a plastic polymer such as; and latex such as
latex beads.

Yet another interesting way of modulating an immune response
10 is to include the immunogen (optionally together with adju-
vants and pharmaceutically acceptable carriers and vehicles)
in a "virtual lymph node" (VLN) (a proprietary medical device
developed by ImmunoTherapy, Inc., 360 Lexington Avenue, New
York, NY 10017-6501). The VLN (a thin tubular device) mimics
15 the structrue and function of a lymph node. Insertion of a VLN
under the skin creates a site of sterile inflammation with an
upsurge of cytokines and chemokines. T- and B-cells as well as
APCs rapidly respond to the danger signals, home to the in-
flamed site and accumulate inside the porous matrix of the
20 VLN. It has been shown that the necessary antigen dose re-
quired to mount an immune response to an antigen is reduced
when using the VLN and that immune protection conferred by
vaccination using a VLN surpassed conventional immunization
using Ribi as an adjuvant. The technology is _i.a._ described
25 briefly in Gelber C _et al._, 1998, "Elicitation of Robust Cel-
lular and Humoral Immune Responses to Small Amounts of Immuno-
gens Using a Novel Medical Device Designated the Virtual Lymph
Node", in: "From the Laboratory to the Clinic, Book of Ab-
stracts, October 12th - 15th 1998, Seascape Resort, Aptos, Cali-
30 fornia".

Microparticle formulation of vaccines has been shown in many cases to increase the immunogenicity of protein antigens and is therefore another preferred embodiment of the invention. Microparticles are made either as co-formulations of antigen

5   with a polymer, a lipid, a carbohydrate or other molecules suitable for making the particles, or the microparticles can be homogeneous particles consisting of only the antigen it- self.

Examples of polymer based microparticles are PLGA and PVP

10 based particles (Gupta, R.K. et. al. 1998) where the polymer and the antigen are condensed into a solid particle. Lipid based particles can be made as micelles of the lipid (so- called liposomes) entrapping the antigen within the micelle (Pietrobon, P.J. 1995). Carbohydrate based particles are typi-

15 cally made of a suitable degradable carbohydrate such as starch or chitosan. The carbohydrate and the antigen are mixed and condensed into particles in a process similar to the one used for polymer particles (Kas, H.S. et. al. 1997).

Particles consisting only of the antigen can be made by vari-

20 ous spraying and freeze-drying techniques. Especially suited for the purporses of the present invention is the super criti- cal fluid technology that is used to make very uniform parti- cles of controlled size (York, P. 1999 & Shekunov, B. et. al. 1999).

25 It is expected that the vaccine should be administered 1-6 times per year, such as 1, 2, 3, 4, 5, or 6 times a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred au- tovaccines according to the invention is not permanent, and

30 therefore the immune system needs to be periodically chal-

lenged with the amyloidogenic polypeptide or modified amyloi-
dogenic polypeptides.

Due to genetic variation, different individuals may react with
immune responses of varying strength to the same polypeptide.
5   Therefore, the vaccine according to the invention may comprise
several different polypeptides in order to increase the immune
response, cf. also the discussion above concerning the choice
of foreign T-cell epitope introductions. The vaccine may com-
prise two or more polypeptides, where all of the polypeptides
10 are as defined above.

The vaccine may consequently comprise 3-20 different modified
or unmodified polypeptides, such as 3-10 different polypep-
tides.

Nucleic acid vaccination

15 As an alternative to classic administration of a peptide-based
vaccine, the technology of nucleic acid vaccination (also
known as "nucleic acid immunisation", "genetic immunisation",
and "gene immunisation") offers a number of attractive fea-
tures.

20 First, in contrast to the traditional vaccine approach, nu-
cleic acid vaccination does not require resource consuming
large-scale production of the immunogenic agent (e.g. in the
form of industrial scale fermentation of microorganisms pro-
ducing modified amyloidogenic polypeptides). Furthermore,
25 there is no need to device purification and refolding schemes
for the immunogen. And finally, since nucleic acid vaccination
relies on the biochemical apparatus of the vaccinated indivi-
dual in order to produce the expression product of the nucleic
acid introduced, the optimum post-translational processing of

56

the expression product is expected to occur; this is espe-
cially important in the case of autovaccination, since, as
mentioned above, a significant fraction of the original B-cell
epitopes should be preserved in the modified molecule, and
5 since B-cell epitopes in principle can be constituted by parts
of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.).
Therefore, native glycosylation and lipidation patterns of the
immunogen may very well be of importance for the overall immu-
nogenicity and this is best ensured by having the host produ-
10 cing the immunogen.

Hence, a preferred embodiment of the invention's variants a-c
comprises effecting presentation of the analogue to the immune
system by introducing nucleic acid(s) encoding the analogue
into the animal's cells and thereby obtaining *in vivo* expres-
15 sion by the cells of the nucleic acid(s) introduced.

In this embodiment, the introduced nucleic acid is preferably
DNA which can be in the form of naked DNA, DNA formulated with
charged or uncharged lipids, DNA formulated in liposomes, DNA
included in a viral vector, DNA formulated with a transfec-
20 tion-facilitating protein or polypeptide, DNA formulated with
a targeting protein or polypeptide, DNA formulated with Cal-
cium precipitating agents, DNA coupled to an inert carrier
molecule, DNA encapsulated in a polymer, e.g. in PLGA (cf. the
microencapsulation technology described in WO 98/31398) or in
25 chitin or chitosan, and DNA formulated with an adjuvant. In
this context it is noted that practically all considerations
pertaining to the use of adjuvants in traditional vaccine for-
mulation apply for the formulation of DNA vaccines. Hence, all
disclosures herein which relate to use of adjuvants in the
30 context of polypeptide based vaccines apply *mutatis mutandis*
to their use in nucleic acid vaccination technology.

As for routes of administration and administration schemes of polypeptide based vaccines which have been detailed above, these are also applicable for the nucleic acid vaccines of the invention and all discussions above pertaining to routes of

5   administration and administration schemes for polypeptides apply *mutatis mutandis* to nucleic acids. To this should be added that nucleic acid vaccines can suitably be administered intraveneously and intraarterially. Furthermore, it is well-known in the art that nucleic acid vaccines can be administered by

10 use of a so-called gene gun, and hence also this and equivalent modes of administration are regarded as part of the present invention. Finally, also the use of a VLN in the administration of nucleic acids has been reported to yield good results, and therefore this particular mode of administration is

15 particularly preferred.

Furthermore, the nucleic acid(s) used as an immunization agent can contain regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A

20 preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitope is produced as a fusion partner to the

25 immunomodulator. Alternatively, two distinct nucleotide fragments can be used, but this is less preferred because of the advantage of ensured co-expression when having both coding regions included in the same molecule.

Accordingly, the invention also relates to a composition for

30 inducing production of antibodies against APP or Aβ, the composition comprising

58

- a nucleic acid fragment or a vector of the invention (cf. the discussion of vectors below), and

- a pharmaceutically and immunologically acceptable vehicle and/or carrier and/or adjuvant as discussed above.

5   Under normal circumstances, the variant-encoding nucleic acid is introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formu-
10 lation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al, 1997, Annu. Rev. Immunol. **15**: 617-648 and Donnelly JJ et al., 1997, Life Sciences **60**: 163-172. Both of these references are incorporated by reference herein.

Live vaccines

15 A third alternative for effecting presentation of the ana-logues as these are defined in variants a-c to the immune sys-tem is the use of live vaccine technology. In live vaccina-tion, presentation to the immune system is effected by admini-stering, to the animal, a non-pathogenic microorganism which
20 has been transformed with a nucleic acid fragment encoding an analogue or with a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by
25 recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella*, etc. Reviews dealing with prepara-tion of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. **45**: 1492-1496 and Walker PD, 1992,
30 Vaccine **10**: 977-990, both incorporated by reference herein.

For details about the nucleic acid fragments and vectors used
in such live vaccines, cf. the discussion below.

As an alternative to bacterial live vaccines, the nucleic acid
fragment of the invention discussed below can be incorporated

5   in a non-virulent viral vaccine vector such as a vaccinia
strain or any other suitable poxvirus.

Normally, the non-pathogenic microorganism or virus is admini-
stered only once to the animal, but in certain cases it may be
necessary to administer the microorganism more than once in a

10 lifetime in order to maintain protective immunity. It is even
contemplated that immunization schemes as those detailed above
for polypeptide vaccination will be useful when using live or
virus vaccines.

Alternatively, live or virus vaccination is combined with pre-

15 vious or subsequent polypeptide and/or nucleic acid vaccina-
tion. For instance, it is possible to effect primary immuniza-
tion with a live or virus vaccine followed by subsequent
booster immunizations using the polypeptide or nucleic acid
approach.

20 The microorganism or virus can be transformed with nucleic
acid(s) containing regions encoding the 1st, 2nd and/or 3rd
moieties, e.g. in the form of the immunomodulating substances
described above such as the cytokines discussed as useful ad-
juvants. A preferred version of this embodiment encompasses

25 having the coding region for the analogue and the coding re-
gion for the immunomodulator in different reading frames or at
least under the control of different promoters. Thereby it is
avoided that the analogue or epitopes are produced as fusion
partners to the immunomodulator. Alternatively, two distinct

30 nucleotide fragments can be used as transforming agents. Of

course, having the 1st and/or 2nd and/or 3rd moieties in the
same reading frame can provide as an expression product, an
analogue of the invention, and such an embodiment is espe-
cially preferred according to the present invention.

Use of the method of the invention in disease treatment

As will be appreciated from the discussions above, the provi-
sion of the method of the invention allows for control of dis-
eases characterized by amyloid deposits. In this context, AD
is the key target for the inventive method but also other dis-
eases characterized by Aβ containing amyloid deposits are fea-
sible targets. Hence, an important embodiment of the method of
the invention for down-regulating amyloid activity comprises
treating and/or preventing and/or ameliorating AD or other
diseases characterized by amyloid deposition, the method com-
prising down-regulating APP or Aβ according to the method of
the invention to such an extent that the amount of amyloid is
significantly decreased.

It is especially preferred that the reduction in amyloid re-
sults in an inversion of the balance between amyloid formation
and amyloid degradation/removal, i.e. that the rate of amyloid
degradation/removal is brought to exceed the rate of amyloid
formation. By carefully controlling the number and immunologi-
cal impact of immunizations of the individual in need thereof
it will be possible to obtain a balance over time which re-
sults in a net reduction of amyloid deposits without having
excessive adverse effects.

Alternatively, if in an individual the method of the invention
cannot remove or reduce existing amyloid deposits, the method
of the invention can be used to obtain a clinically signifi-

61

cant reduction in the formation of new amyloid, thereby sig-
nificantly prolonging the time where the disease condition is
non-debilitating. It should be possible to monitor the rate of
amyloid depositing by either measuring the serum concentration

5 of amyloid (which is believed to be in equilibrium with the
deposited material), or by using positron-emission tomography
(PET) scanning, cf. Small GW, *et al.*, 1996, Ann N Y Acad Sci
802: 70-78.

Other diseases and conditions where the present means and
10 methods may be used in treatment or amelioration in an analo-
gous way have been mentioned above in the "Background of the
invention" or are listed below in the section headed "other
amyloidic diseases and proteins associated therewith".

Peptides, polypeptides, and compositions of the invention

15 As will be apparent from the above, the present invention is
based on the concept of immunising individuals against the APP
or Aβ antigen in order to obtain a reduced amount of pathology-
related amyloid deposits. The preferred way of obtaining such
an immunization is to use the analogues described herein,
20 thereby providing molecules which have not previously been
disclosed in the art.

It is believed that the analogues discussed herein are inven-
tive in their own right, and therefore an important part of
the invention pertains to an analogue as described above.
25 Hence, any disclosure presented herein pertaining to modified
APP or Aβ are relevant for the purpose of describing the amy-
loidogenic analogues of the invention, and any such disclo-
sures apply *mutatis mutandis* to the description of these ana-
logues.

It should be noted that preferred modified APP or Aβ molecules comprise modifications which results in a polypeptide having a sequence identity of at least 70% with APP or Aβ or with a sub-sequence thereof of at least 10 amino acids in length. Higher

5 sequence identities are preferred, e.g. at least 75% or even at least 80, 85, 90, or 95%. The sequence identity for pro-teins and nucleic acids can be calculated as $(N_{ref} - N_{dif}) \cdot 100/N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is

10 the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N_{dif}=2 and N_{ref}=8).

The invention also pertains to compositions useful in exerci-sing the method of the invention. Hence, the invention also

15 relates to an immunogenic composition comprising an immuno-genically effective amount of an analouge as described above, said composition further comprising a pharmaceutically and im-munologically acceptable diluent and/or vehicle and/or carrier and/or excipient and optionally an adjuvant. In other words,

20 this part of the invention concerns formulations of analogues, essentially as described above. The choice of adjuvants, car-riers, and vehicles is accordingly in line with what has been discussed above when referring to formulation of modified and unmodified amyloidogenic polypeptide for use in the inventive

25 method for the down-regulation of APP or Aβ.

The polypeptides are prepared according to methods well-known in the art. Longer polypeptides are normally prepared by means of recombinant gene technology including introduction of a nu-cleic acid sequence encoding the analogue into a suitable vec-

30 tor, transformation of a suitable host cell with the vector, expression by the host cell of the nucleic acid sequence, re-

covery of the expression product from the host cells or their
culture supernatant, and subseqeunt purification and optional
further modification, e.g. refolding or derivatization.

Shorter peptides are preferably prepared by means of the well-
5   known techniques of solid- or liquid-phase peptide synthesis.
However, recent advances in this technology has rendered
possible the production of full-length polypeptides and pro-
teins by these means, and therefore it is also within the
scope of the present invention to prepare the long constructs
10   by synthetic means.

Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that poly-
amino acid analogues can be prepared by means of recombinant
gene technology but also by means of chemical synthesis or
15   semisynthesis; the latter two options are especially relevant
when the modification consists in coupling to protein carriers
(such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and
non-proteinaceous molecules such as carbohydrate polymers and
of course also when the modification comprises addition of
20   side chains or side groups to an APP or Aβ derived peptide
chain.

For the purpose of recombinant gene technology, and of course
also for the purpose of nucleic acid immunization, nucleic
acid fragments encoding analogues are important chemical pro-
25   ducts. Hence, an important part of the invention pertains to a
nucleic acid fragment which encodes an analogue of the inven-
tion, i.e. an APP or Aβ derived polypeptide which either com-
prises the natural sequence to which has been added or in-
serted a fusion partner or, preferably an APP or Aβ derived

polypeptide wherein has been introduced a foreign T-cell epitope by means of insertion and/or addition, preferably by means of substitution and/or deletion. The nucleic acid fragments of the invention are either DNA or RNA fragments.

5   The nucleic acid fragments of the invention will normally be inserted in suitable vectors to form cloning or expression vectors carrying the nucleic acid fragments of the invention; such novel vectors are also part of the invention. Details concerning the construction of these vectors of the invention
10   will be discussed in context of transformed cells and microorganisms below. The vectors can, depending on purpose and type of application, be in the form of plasmids, phages, cosmids, mini-chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector.
15   Preferred cloning and expression vectors of the invention are capable of autonomous replication, thereby enabling high copy-numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the
20   following features in the 5'→3' direction and in operable linkage: a promoter for driving expression of the nucleic acid fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling secretion (to the extracellular phase or, where applicable, into the periplasma) of or
25   integration into the membrane of the polypeptide fragment, the nucleic acid fragment of the invention, and optionally a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell pre-
30   ferred that the vector when introduced into a host cell is integrated in the host cell genome. In contrast, when working

with vectors to be used for effecting *in vivo* expression in an
animal (*i.e.* when using the vector in DNA vaccination) it is
for security reasons preferred that the vector is incapable of
being integrated in the host cell genome; typically, naked DNA
5   or non-integrating viral vectors are used, the choices of
which are well-known to the person skilled in the art

The vectors of the invention are used to transform host cells
to produce the analogue of the invention. Such transformed
cells, which are also part of the invention, can be cultured
10   cells or cell lines used for propagation of the nucleic acid
fragments and vectors of the invention, or used for recombi-
nant production of the analogues of the invention. Alterna-
tively, the transformed cells can be suitable live vaccine
strains wherein the nucleic acid fragment (one single or mul-
15   tiple copies) have been inserted so as to effect secretion or
integration into the bacterial membrane or cell-wall of the
analogue.

Preferred transformed cells of the invention are microorga-
nisms such as bacteria (such as the species *Escherichia* [e.g.
20   *E.coli*], *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or *My-
cobacterium* [preferably non-pathogenic, e.g. *M. bovis* BCG]),
yeasts (such as *Saccharomyces cerevisiae*), and protozoans. Al-
ternatively, the transformed cells are derived from a multi-
cellular organism such as a fungus, an insect cell, a plant
25   cell, or a mammalian cell. Most preferred are cells derived
from a human being, cf. the discussion of cell lines and vec-
tors below. Recent results have shown great promise in the use
of a commercially available *Drosophila melanogaster* cell line
(the Schneider 2 (S$_2$) cell line and vector system available
30   from Invitrogen) for the recombinant production of polypep-

tides in applicants' lab, and therefore this expression system
is particularly preferred.

For the purposes of cloning and/or optimized expression it is
preferred that the transformed cell is capable of replicating
5   the nucleic acid fragment of the invention. Cells expressing
the nucleic fragment are preferred useful embodiments of the
invention; they can be used for small-scale or large-scale
preparation of the analogue of the invention or, in the case
of non-pathogenic bacteria, as vaccine constituents in a live
10   vaccine.

When producing the analogues of the invention by means of
transformed cells, it is convenient, although far from essen-
tial, that the expression product is either exported out into
the culture medium or carried on the surface of the trans-
15   formed cell.

When an effective producer cell has been identified it is pre-
ferred, on the basis thereof, to establish a stable cell line
which carries the vector of the invention and which expresses
the nucleic acid fragment encoding the modified amyloidogenic
20   polypeptide. Preferably, this stable cell line secretes or
carries the analogue of the invention, thereby facilitating
purification thereof.

In general, plasmid vectors containing replicon and control
sequences which are derived from species compatible with the
25   host cell are used in connection with the hosts. The vector
ordinarily carries a replication site, as well as marking se-
quences which are capable of providing phenotypic selection in
transformed cells. For example, *E. coli* is typically trans-
formed using pBR322, a plasmid derived from an *E. coli* species
30   (see, e.g., Bolivar *et al.*, 1977). The pBR322 plasmid contains

genes for ampicillin and tetracycline resistance and thus pro-
vides easy means for identifying transformed cells. The pBR
plasmid, or other microbial plasmid or phage must also con-
tain, or be modified to contain, promoters which can be used
5 by the prokaryotic microorganism for expression.

Those promoters most commonly used in recombinant DNA con-
struction include the B-lactamase (penicillinase) and lactose
promoter systems (Chang *et al.*, 1978; Itakura *et al.*, 1977;
Goeddel *et al.*, 1979) and a tryptophan (trp) promoter system
10 (Goeddel *et al.*, 1979; EP-A-0 036 776). While these are the
most commonly used, other microbial promoters have been dis-
covered and utilized, and details concerning their nucleotide
sequences have been published, enabling a skilled worker to
ligate them functionally with plasmid vectors (Siebwenlist *et
al.*, 1980). Certain genes from prokaryotes may be expressed
15 *al.*, 1980). Certain genes from prokaryotes may be expressed
efficiently in *E. coli* from their own promoter sequences, pre-
cluding the need for addition of another promoter by artifi-
cial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast
20 cultures may also be used, and here the promoter should be ca-
pable of driving expression. *Saccharomyces cerevisiase*, or
common baker's yeast is the most commonly used among eukaryo-
tic microorganisms, although a number of other strains are
commonly available. For expression in *Saccharomyces*, the plas-
25 mid YRp7, for example, is commonly used (Stinchcomb *et al.*,
1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This
plasmid already contains the trpl gene which provides a selec-
tion marker for a mutant strain of yeast lacking the ability
to grow in tryptophan for example ATCC No. 44076 or PEP4-1
30 (Jones, 1977). The presence of the trpl lesion as a characte-
ristic of the yeast host cell genome then provides an effec-

tive environment for detecting transformation by growth in the
absence of tryptophan.

Suitable promoting sequences in yeast vectors include the pro-
moters for 3-phosphoglycerate kinase (Hitzman *et al.*, 1980) or
5 other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*,
1978), such as enolase, glyceraldehyde-3-phosphate dehydro-
genase, hexokinase, pyruvate decarboxylase, phosphofructo-
kinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mu-
tase, pyruvate kinase, triosephosphate isomerase, phosphoglu-
10 cose isomerase, and glucokinase. In constructing suitable ex-
pression plasmids, the termination sequences associated with
these genes are also ligated into the expression vector 3' of
the sequence desired to be expressed to provide polyadenyla-
tion of the mRNA and termination.

15 Other promoters, which have the additional advantage of tran-
scription controlled by growth conditions are the promoter re-
gion for alcohol dehydrogenase 2, isocytochrome C, acid phos-
phatase, degradative enzymes associated with nitrogen metabo-
lism, and the aforementioned glyceraldehyde-3-phosphate dehy-
20 drogenase, and enzymes responsible for maltose and galactose
utilization. Any plasmid vector containing a yeast-compatible
promoter, origin of replication and termination sequences is
suitable.

In addition to microorganisms, cultures of cells derived from
25 multicellular organisms may also be used as hosts. In princip-
le, any such cell culture is workable, whether from vertebrate
or invertebrate culture. However, interest has been greatest
in vertebrate cells, and propagation of vertebrate in culture
(tissue culture) has become a routine procedure in recent
30 years (Tissue Culture, 1973). Examples of such useful host

cell lines are VERO and HeLa cells, Chinese hamster ovary
(CHO) cell lines, and W138, BHK, COS-7 293, *Spodoptera
frugiperda* (SF) cells (commercially available as complete ex-
pression systems from *i.a.* Protein Sciences, 1000 Research
5 Parkway, Meriden, CT 06450, U.S.A. and from Invitrogen), and
MDCK cell lines. In the present invention, an especially pre-
ferred cell line is S_2 available from Invitrogen, PO Box 2312,
9704 CH Groningen, The Netherlands.

Expression vectors for such cells ordinarily include (if
10 necessary) an origin of replication, a promoter located in
front of the gene to be expressed, along with any necessary
ribosome binding sites, RNA splice sites, polyadenylation
site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the ex-
15 pression vectors are often provided by viral material. For ex-
ample, commonly used promoters are derived from polyoma, Ade-
novirus 2, and most frequently Simian Virus 40 (SV40). The
early and late promoters of SV40 virus are particularly useful
because both are obtained easily from the virus as a fragment
20 which also contains the SV40 viral origin of replication
(Fiers *et al.*, 1978). Smaller or larger SV40 fragments may
also be used, provided there is included the approximately 250
bp sequence extending from the *Hind*III site toward the *Bgl*I
site located in the viral origin of replication. Further, it
25 is also possible, and often desirable, to utilize promoter or
control sequences normally associated with the desired gene
sequence, provided such control sequences are compatible with
the host cell systems.

An origin of replication may be provided either by construc-
30 tion of the vector to include an exogenous origin, such as may

be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

5   Identification of useful analogues

It will be clear to the skilled person that not all possible variants or modifications of naturally occurring APP or Aβ will have the ability to elicit antibodies in an animal which are cross-reactive with the natural form. It is, however, not dif-
10 ficult to set up an effective standard screen for modified amyloidogenic molecules which fulfill the minimum requirements for immunological reactivity discussed herein. Hence, it is possible to utilise a method for the identification of a modified amyloidogenic polypeptide which is capable of inducing
15 antibodies against unmodified amyloidogenic polypeptide in an animal species where the unmodified amyloidogenic polypeptide is a (non-immunogenic) self-protein, the method comprising

-      preparing, by means of peptide synthesis or genetic engineering techniques, a set of mutually distinct analogue
20   of the invention wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of an APP or Aβ of the animal species thereby giving rise to amino acid sequences in the set which comprise T-cell epitopes which are foreign to the
25   animal species, or preparing a set of nucleic acid fragments encoding the set of mutually distinct analogues,

-      testing members of the set of analogues or nucleic acid fragments for their ability to induce production of anti-

bodies by the animal species against the unmodified APP
or Aβ, and

- identifying and optionally isolating the member(s) of the
  set of analogues which significantly induces antibody
  production against unmodified APP or Aβ in the species or
  identifying and optionally isolating the polypeptide ex-
  pression products encoded by members of the set of nu-
  cleic acid fragments which significantly induces antibody
  production against unmodified APP or Aβ in the animal spe-
  cies.

In this context, the "set of mutually distinct modified amy-
loidogenic polypeptides" is a collection of non-identical
analouges which have e.g. been selected on the basis of the
criteria discussed above (e.g. in combination with studies of
circular dichroism, NMR spectra, and/or X-ray diffraction pat-
terns). The set may consist of only a few members but it is
contemplated that the set may contain several hundred members.

The test of members of the set can ultimately be performed *in
vivo*, but a number of *in vitro* tests can be applied which nar-
row down the number of modified molecules which will serve the
purpose of the invention.

Since the goal of introducing the foreign T-cell epitopes is
to support the B-cell response by T-cell help, a prerequisite
is that T-cell proliferation is induced by the analogue. T-
cell proliferation can be tested by standardized proliferation
assays *in vitro*. In short, a sample enriched for T-cells is
obtained from a subject and subsequently kept in culture. The
cultured T-cells are contacted with APCs of the subject which
have previously taken up the modified molecule and processed

it to present its T-cell epitopes. The proliferation of T-
cells is monitored and compared to a suitable control (e.g. T-
cells in culture contacted with APCs which have processed in-
tact, native amyloidogenic polypeptide). Alternatively, pro-
5   liferation can be measured by determining the concentration of
relevant cytokines released by the T-cells in response to
their recognition of foreign T-cells.

Having rendered highly probable that at least one analogue of
either type of set is capable of inducing antibody production
10 against APP or Aβ, it is possible to prepare an immunogenic
composition comprising at least one analouge which is capable
of inducing antibodies against unmodified APP or Aβ in an ani-
mal species where the unmodified APP or Aβ is a self-protein,
the method comprising admixing the member(s) of the set which
15 significantly induces production of antibodies in the animal
species which are reactive with the APP or Aβ with a pharmaceu-
tically and immunologically acceptable carrier and/or vehicle
and/or diluent and/or excipient, optionally in combination
with at least one pharmaceutically and immunologically accept-
20 able adjuvant.

The above-described tests of polypeptide sets are conveniently
carried out by initially preparing a number of mutually dis-
tinct nucleic acid sequences or vectors of the invention, in-
serting these into appropriate expression vectors, transform-
25 ing suitable host cells (or host animals) with the vectors,
and effecting expression of the nucleic acid sequences of the
invention. These steps can be followed by isolation of the ex-
pression products. It is preferred that the nucleic acid se-
quences and/or vectors are prepared by methods comprising ex-
30 ercise of a molecular amplification technique such as PCR or
by means of nucleic acid synthesis.

73

Specific amyloidogenic targets

In addition to the proteins most often associated with Alzhei-
mer's, APP, ApoE4 and Tau, there is long list of other pro-
teins that have somehow been linked to AD, either by their di-
5   rect presence in plaques or tangles of AD brains or by their
apparent genetic association with increased risk of developing
AD. Most, if not all, of these antigens are together with the
above-discussed Aβ, APP, presenilin and ApoE4, putative target
proteins in certain embodiment of the present invention. These
10 putative targets are already discussed thoroughly in WO
01/62284. Hence, these putative targets will only be mentioned
briefly here, whereas a more thorough background discussion
can be be found in WO 01/62282 which is hereby incorporated by
reference herein:

15 Alpha1-antichymotrypsin (ACT); Alpha2-macroglobulin; ABAD (Aβ-
peptide binding alcohol dehydrogenase); APLP1 and -2 (amyloid
precursor like protein 1 and -2); AMY117; Bax; Bcl-2; Bleomy-
cin hydrolase; BRI/ABRI; Chromogranin A; Clusterin/apoJ; CRF
(corticotropin releasing factor) binding protein; EDTF (endo-
20 thelial-derived toxic factor); Heparan sulfate proteoglycans;
Human collapsin response mediator protein-2; Huntingtin (Hun-
tington's disease protein); ICAM-I; IL-6; Lysosome-associated
antigen CD68; P21 ras; PLC-delta 1 (phospholipase C isoenzyme
delta 1); Serum amyloid P component (SAP); Synaptophysin;
25 Synuclein (alpha-synuclein or NACP); and TGF-b1 (transforming
growth factor b1).

The presently described means and methods for down-regulation
of APP or Aβ can be combined with therapies, e.g. active spe-
cific immunotherapy, against any of these other amyloidogenic
30 polypeptides.

74

Apart from Alzheimer's disease, also cerebral amyloid angiopathy is a disease that would be a suitable target for the presently disclosed technology.

It is contemplated that most methods for immunizing against
5   APP or Aβ should be restricted to immunization giving rise to antibodies cross-reactive with the native APP or Aβ. Nevertheless, in some cases it will be of interest to induce cellular immunity in the form of CTL responses against cells which present MHC Class I epitopes from the amyloidogenic polypeptides
10 - this can be expedient in those cases wherein reduction in the number of cells producing APP or Aβ does not constitute a serious adverse effect. In such cases where CTL responses are desired it is preferred to utilise the teachings of Applicant's WO 00/20027. The disclosures of these two documents are
15 hereby incorporated by reference herein.

IMMUNOGEN CARRIERS

Molecules comprising a T helper epitope and APP or Aβ peptides representing or including B-cell epitopes linked covalently to a non-immunogenic polymer molecule acting as a vehicle, e.g. a
20 multivalent activated poly-hydroxypolymer, will, as mentioned above, function as a vaccine molecule that only contains the immunologically relevant parts, can be obtained, and are interesting embodiments in variants d and e disclosed above. Promiscuous or so-called universal T-helper epitopes can be
25 used if e.g. the target for the vaccine is a self-antigen such as APP or Aβ. Furthermore, elements that enhance the immunological response could be also co-coupled to the vehicle and thereby act as an adjuvant. Such elements could be mannose, tuftsin, muramyl dipeptide, CpG motifs etc. In that case, sub-

75

sequent adjuvant formulation of the vaccine product might be unnecessary and the product could be administered in pure water or saline.

By coupling cytotoxic T cell (CTL) epitopes together with the
5   T-helper epitopes it will also be possible to generate CTL's specific for the antigen from which the CTL epitope was derived. Elements that promote uptake of the product to the cytosol, such as mannose, of the APC, e.g. a macrophage, could also be co-coupled to the vehicle together with the CTL- and
10 the T helper epitope and enhance the CTL response.

The ratio of B-cell epitopes and T-helper epitopes (P2 and P30) in the final product can be varied by varying the concentration of these peptides in the synthesis step. As mentioned above, the immunogenic molecule can be tagged with e.g. man-
15 nose, tuftsin, CpG-motifs or other immune stimulating substances (described herein) by adding these, if necessary by using e.g. aminated derivatives of the substances, to the carbonate buffer in the synthesis step.

If an insoluble activated polyhydroxy polymer is used to com-
20 bine the peptides containing the APP or Aβ B-cell epitope and the T-helper epitopes it can, as mentioned above be performed as a solid phase synthesis and the final product can be harvested and purified by wash and filtration. The elements to be coupled to a tresyl activated polyhydroxypolymer (peptides,
25 tags etc) can be added to the polyhydroxypolymer at low pH, e.g. pH 4-5, and allowed to be equally distributed in the "gel" by passive diffusion. Subsequently, the pH can be raised to pH 9-10 to start the reaction of the primary amino groups on the peptides and tags to the tresyl groups on the polyhy-
30 droxy polymer. After coupling of peptides and e.g. immune

stimulating elements the gel is grinded to form particles of
suitable size for immunization.

Such an immunogen therefore comprises

   a)   at least one first amino acid sequence derived from
5   APP or Aβ, wherein the at least one first amino
             acid sequence contains at least one B-cell and/or
             at least one CTL epitope, and

   b)   at least one second amino acid sequence that in-
             cludes a foreign T helper cell epitope,

10 wherein each of the at least first and at least second amino
   acid sequences are coupled to a pharmaceutically acceptable
   activated polyhydroxypolymer carrier.

   In order for the amino acid sequences to couple to the polyhy-
   droxypolymer it is normally necessary to "activate" the poly-
15 hydroxypolymer with a suitable reactive group that can form
   the necessary link to the amino acid sequences.

   The term "polyhydroxypolymer" is intended to have the same
   meaning as in WO 00/05316, i.e. the polyhydroxypolymer can
   have exactly the same characteristics as is specifically
20 taught in that application. Hence, the polyhydroxypolymer can
   be water soluble or insoluble (thus requiring different syn-
   thesis steps during preparation of the immunogen). The polyhy-
   droxypolymer can be selected from naturally occurring polyhy-
   droxy compounds and synthetic polyhydroxy compounds.

25 Specific and preferred polyhydroxypolymers are polysaccharides
   selected from acetan, amylopectin, gum agar-agar, agarose,
   alginates, gum Arabic, carregeenan, cellulose, cyclodextrins,

dextran, furcellaran, galactomannan, gelatin, ghatti, glucan, glycogen, guar, karaya, konjac/A, locust bean gum, mannan, pectin, psyllium, pullulan, starch, tamarine, tragacanth, xanthan, xylan, and xyloglucan. Dextran is especially preferred.

5 However, the polyhydroxypolymer can also be selected from highly branched poly(ethyleneimine)(PEI), tetrathienylene vinylene, Kevlar (long chains of poly-paraphenyl terephtalamide), Poly(urethanes), Poly(siloxanes), polydimethylsiloxane, silicone, Poly(methyl methacrylate) (PMMA), Poly(vinyl alco-

10 hol), Poly(vinyl pyrrolidone), Poly(2-hydroxy ethyl methacrylate), Poly(N-vinyl pyrrolidone), Poly(vinyl alcohol), Poly(acrylic acid), Polytetrafluoroethylene (PTFE), Polyacrylamide, Poly(ethylene-co-vinyl acetate), Poly(ethylene glycol) and derivatives, Poly(methacrylic acid), Polylactides

15 (PLA), Polyglycolides (PGA), Poly(lactide-co-glycolides) (PLGA), Polyanhydrides, and Polyorthoesters.

The (weight) average molecular weight of the polyhydroxypolymer in question (i.e. before activation) is typically at least 1,000, such as at least 2,000, preferably in the range of

20 2,500-2,000,000, more preferably in the range of 3,000-1,000,000, in particular in the range of 5,000-500,000. It has been shown in the examples that polyhydroxypolymers having an average molecular weight in the range of 10,000-200,000 are particularly advantageous.

25 The polyhydroxypolymer is preferably water soluble to an extent of at least 10 mg/ml, preferably at least 25 mg/ml, such as at least 50 mg/ml, in particular at least 100 mg/ml, such as at least 150 mg/ml at room temperature. It is known that dextran, even when activated as described herein, fulfils the

30 requirements with respect to water solubility.

78

For some of the most interesting polyhydroxypolymers, the ra-
tio between C (carbon atoms) and OH groups (hydroxy groups) of
the unactivated polyhydroxypolymers (i.e. the native polyhy-
droxypolymer before activation) is in the range of 1.3 to 2.5,
5   such as 1.5-2.3, preferably 1.6-2.1, in particular 1.85-2.05.
Without being bound to any specific theory, it is believed
that such as a C/OH ratio of the unactivated polyhydroxypoly-
mer represents a highly advantageous level of hydrophilicity.
Polyvinylalcohol and polysaccharides are examples of polyhy-
10 droxypolymers which fulfil this requirement. It is believed
that the above-mentioned ratio should be roughly the same for
the activated polyhydroxypolymer as the activation ratio
should be rather low.

The term "polyhydroxypolymer carrier" is intended to denote
15 the part of the immunogen that carries the amino acid se-
quences. As a general rule, the polyhydroxypolymer carrier has
its outer limits where the amino acid sequences can be cleaved
of by a peptidase, e.g. in an antigen presenting cell that is
processing the immunogen. Hence, the polyhydroxypolymer car-
20 rier can be the polyhydroxypolymer with an activation group,
where the bond between the activation group and the amino acid
sequence is cleavable by a peptidase in an APC, or the polyhy-
droxypolymer carrier can be a polyhydroxypolymer with activa-
tion group and e.g. a linker such as a single L-amino acid or
25 a number of D-amino acids, where the last part of the linker
can bond to the amino acid sequences and be cleaved by a pep-
tidase in an APC.

As mentioned above, the polyhydroxypolymers carry functional
groups (activation groups), which facilitate the anchoring of
30 peptides to the carrier. A wide range of applicable functional
groups are known in the art, e.g. tresyl (trifluoroethylsul-

phonyl), maleimido, p-nitrophenyl cloroformate, cyanogenbro-
mide, tosyl (p-toluenesulfonyl), triflyl (trifluoromethanesul-
fonyl), pentafluorobenzenesulfonyl, and vinyl sulphone groups.
Preferred examples of functional groups within the present in-
5   vention are tresyl, maleimido, tosyl, triflyl, pentafluoroben-
zenesulfonyl, p-nitrophenyl cloroformate, and vinylsulphone
groups, among which tresyl, maleimido, and tosyl groups are
particularly relevant.

Tresyl activated polyhydroxypolymers can be prepared using
10 tresyl chloride as described for activation of dextran in Ex-
ample 1 in WO 00/05316 or as described in Gregorius et al., J.
Immunol. Meth. 181 (1995) 65-73.

Maleimido activated polyhydroxypolymers can be prepared using
p-maleimidophenyl isocyanate as described for activation of
15 dextran in Example 3 of WO 00/05316. Alternatively, maleimido
groups could be introduced to a polyhydroxypolymer, such as
dextran, by derivatisation of a tresyl activated polyhydroxy-
polymer (such as tresyl activated dextran (TAD)) with a dia-
mine compound (generally $H_2N-C_nH_{2n}-NH_2$, where n is 1-20, pre-
20 ferably 1-8), e.g. 1,3-diaminopropane, in excess and subse-
quently react the amino groups introduced in TAD with reagents
such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-car-
boxylate (SMCC), sulfo-succinimidyl 4-(N-maleimidomethyl)-
cyclohexane-1-carboxylate (sulfo-SMCC), succinimidyl 4-(p-
25 maleimidophenyl)butyrate (SMPB), sulfo-succinimidyl 4-(p-
maleimidophenyl)butyrate (sulfo-SMPB), N-γ-maleimidobutyryloxy-
succinimide ester (GMBS) or N-γ-maleimidobutyryloxy-sulfosuc-
cinimide ester. Although the different reagents and routes
for activation formally results in slightly different male-
30 imide activated products with respect to the linkage between
the maleimide functionality and the remainder of the parent

hydroxy group on which activation is performed, all and every are considered as "maleimide activated polyhydroxypolymers".

Tosyl activated polyhydroxypolymers can be prepared using tosyl chloride as described for activation of dextran in Example
5 2 in WO 00/05316. Triflyl and pentafluorobenzenesulfonyl activated polyhydroxypolymers are prepared as the tosyl or tresyl activated analogues, e.g. by using the corresponding acid chlorides.

Cyanogenbromide activated polyhydroxypolymer can be prepared
10 by reacting the polyhydroxypolymer with cyanogenbromide using conventional methods. The resulting functional groups are normally cyanate esters with two hydroxy groups of the polyhydroxypolymer.

The degree of activation can be expressed as the ratio between
15 the free hydroxy groups and the activation groups (i.e. functionalised hydroxy groups). It is believed that a ratio between the free hydroxy groups of the polyhydroxypolymer and the activation groups should be between 250:1 and 4:1 in order to obtain an advantageous balance between the hydrophilicity
20 and the reactivity of the polyhydroxypolymer. Preferably the ratio is between 100:1 and 6:1, more preferably between 60:1 and 8:1, in particular between 40:1 and 10:1.

Especially interesting activated polyhydroxypolymers for use in the method for producing the generally applicable immunogen
25 according to the invention are tresyl, tosyl and maleimido activated polysaccharides, especially tresyl activated dextran (TAD), tosyl activated dextran (TosAD), and maleimido activated dextran (MAD).

It is preferred that the bond between the polyhydroxypolymer
carrier and the amino acid sequences attached thereto are
cleavable by a peptidase, e.g. as a peptidase active in the
processing of antigens in an APC. It is therefore preferred
5   that the at least first and at least second amino acid se-
quences are coupled to the activated polyhydroxypolymer car-
rier via an amide bond or a peptide bond. It is especially
preferred that the at least first and at least second amino
acid sequences each provide for the nitrogen moiety of their
10 respective amide bond.

The polyhydroxypolymer carrier may be substantially free of
amino acid residues, necessitating that the activation group
provides for part of a peptidase cleavable bond, but as men-
tioned above, the carrier may also simply include a spacer in-
15 cluding at least one L-amino acid. Nevertheless, the at least
first and at least second amino acid sequences are normally
bound to the activated version of the polyhydroxypolymer via
the nitrogen at the N-terminus of the amino acid sequence.

The above-described generally applicable immunogen of the pre-
20 sent invention can be used in immunization methods essentially
as described herein for polypeptide vaccines. That is, all
disclosures relating to dosages, mode of administration and
formulation of polypeptide vaccines for down-regulating the
amyloidogenic polypeptides discussed herein apply *mutatis mu-*
25 *tandis* to the generally applicable immunogens.

GENERALLY APPLICABLE SAFE VACCINATION TECHNOLOGY

As discussed above, one preferred embodiment of the present
invention entails the use of variants of amyloidogenic poly-
peptides that are incapable of providing self-derived T_H epi-

topes that may drive an immune response against the amyloido-
genic polypeptide.

However, it is believed by the present inventors that this
strategy for designing anti-self vaccines and for effecting
5 anti-self immunity, is a generally applicable technology that
is inventive in its own right. It should prove especially
suited in cases where the self-antigen it is sought to down-
regulate is sufficiently abundant in the body so that it is
possible that self-stimulation of an immune response could
10 happen. Hence, all disclosures above of this embodiment inso-
far as it relates to the provision of an anti-self immune re-
sponse against APP or Aβ applies mutatis mutandis to immuniza-
tion against other self-polypeptides, especially those that
are present in sufficient amounts for them to maintain the im-
15 mune response in the form of an uncontrolled autoimmune condi-
tion because autologous T_H epitopes of the relevant self-poly-
peptide are driving the immune response.

EXAMPLE 1

The Auto Vaccination approach for Immunizing against AD

20 The fact that Aβ protein knock out mice does not show any ab-
normalities or adverse side effects, suggest that removal or
lowering the amounts of Aβ will be safe, Zheng H. (1996).

Published experiments where transgenic animals are immunized
against the transgenic human Aβ protein suggest that if it was
25 possible to break the self tolerance, down-regulation of Aβ
could be obtained by auto-reactive antibodies. These experi-
ments further suggest that such down regulation of Aβ poten-

83

tially would both prevent the formation of plaques, and even clear already formed Aβ plaques from the brain, cf. Schenk et al. (1999). But, traditionally it is not possible to raise antibodies against self-proteins.

5   The published data does thus not provide the means for breaking true self-tolerance towards true self-proteins. Nor does the data provide information on how to ensure that the immune reaction is directed solely or predominantly towards the Aβ deposits, and not towards the cell membrane bound Aβ precursor
10   protein (APP), if this is deemed necessary. An immune response generated using the existing technology would presumably generate an immune response towards self-proteins in an unregulated way so unwanted and excessive auto-reactivity towards parts the Aβ protein may be generated. Hence, using existing
15   immunization strategies will most likely be unable to generate strong immune responses towards self-proteins and will furthermore be unsafe due to potential strong cross-reactivity towards membrane bound APP which is present on a large number of cells in the CNS.

20   The present invention provides the means of effectively generating a strong regulated immune response towards true self-proteins which potentially could form plaques and cause serious disease in the CNS or in other compartments of the body. A safe and efficacious human Aβ protein therapeutic vac-
25   cine will be developed by using this technology for the treatment of AD.

In light of this, it is possible to anticipate that AD, a disease predicted to cripple the health care system in the next century, could be cured, or such vaccines described could at
30   least constitute an effective therapeutical approach for

84

treatment of the symptoms and progression of this disease. This technique represents a entirely new immunological approach to blocking amyloid deposition in AD and other neurologic diseases as well.

5   In the following table, 35 contemplated constructs are indicated. All positions given in the table are relative to the starting Methionine of APP (first amino acid in SEQ ID NO: 2) and include both the starting and ending amino acid, e.g. the 672 - 714 fragment includes both amino acid 672 and 714. The

10 starting and ending positions for P2 and P30 indicate that the epitope substitutes a part of the APP fragment at the positions indicated (both positions included in the substitution) - in most constructs, the introduced epitopes substitutes a fragment of the length of the epitope. The asterisks in the

15 table have the following meaning:

*)   Only one position for P2 and P30 indicates that the epitope has been *inserted* into the APP derivative at the position indicated (the epitope begins at the amino acid C-terminally adjacent to the given posi-

20   tion).

**)   Construction 34 contains three identical APP fragments separated by P30 and P2, respectively.

***)   Construction 35 contains nine identical APP fragments separated by alternating P30 and P2

25   epitopes.

APP AutoVac constructions

Var. No.	Start of APP segment relative to aa 1 of APP	End of APP segment relative to aa 1 of APP	Position of P2 epitope relative to aa 1 of APP	Position of P30 epitope relative to aa 1 of APP	Molecule length
1	630	770	656 – 670	635 – 655	141
2	630	714	656 – 670	635 – 655	85
3	672	770	735 – 749	714 – 728	99
4	672	770		714 – 728	99
5	672	770	714 – 728		99
6	672	770	723*	723*	135
7	672	770		723*	120
8	672	770	723*		114
9	672	714		672*	64
10	672	714		714*	64
11	672	714	672*		58
12	672	714	714*		58
13	672	714	714*	672*	79
14	672	714	680 – 694		43
14	672	714	685 – 799		43
16	672	714	690 – 704		43
17	672	714	695 – 709		43
18	672	714		675 – 695	43
19	672	714		680 – 700	43
20	672	714		685 – 705	43
21	672	714		690 – 710	43
22	672	714	680*	680*	79
23	672	714	690*	690*	79
24	672	714	700*	700*	79
25	672	714	710*	710*	79
26	672	714		680*	64
27	672	714		690*	64
28	672	714		700*	64
29	672	714		710*	64
30	672	714	680*		58
31	672	714	690*		58
32	672	714	700*		58
33	672	714	710*		58
34	672	714	After rep. 1**	After rep. 2**	165
35	672	714	34 x 3*	34 x 3***	165

The part of APP, against which it most interesting to generate a response, is the 43 amino acid Aβ core peptide (Aβ-43, corresponding to SEQ ID NO: 2, residues 672-714) that is the main constituent of amyloid plaques in AD brains. This APP fragment is part of all constructions listed above.

Variants 1 and 2 comprise a portion of APP upstream of Aβ-43 where the model epitopes P2 and P30 have been placed. Variants 1 and 3-8 all comprise the C-100 fragment which has been shown to be neurotoxic – the C-100 fragment corresponds to amino acid residues 714-770 of SEQ ID NO: 2. In variants 3-5 the epitopes substitutes a part of the C-100 fragment while the in variants 6-8 have been inserted into C-100.

Variants 9-35 contain only the core Aβ-43 protein. In variants 9-13, P2 and P30 are fused to either end of Aβ-43; in 14-21 P2 and P30 substitutes part of Aβ-43; in 22-33 P2 and P30 are inserted into Aβ-43; 34 contains three identical Aβ-43 fragments spaced by P30 and P2, respectively; 35 contains 9 Aβ-43 repeats spaced by alternating P2 and P30 epitopes.

Truncated parts of the above-discussed Aβ-43 protein can also be employed in immunogenic analogues according to the present invention. Especially preferred are the truncates Aβ(1-42), Aβ(1-40), Aβ(1-39), Aβ(1-35), Aβ(1-34), Aβ(1-34), Aβ(1-28), Aβ(1-12), Aβ(1-5), Aβ(13-28), Aβ(13-35), Aβ(17-28), Aβ(25-35), Aβ(35-40), Aβ(36-42), and Aβ(35-42) (where the numbers in the parentheses indicate the amino acid stretches of Aβ-43 that constitute the relevant fragment – Aβ(35-40) is e.g. identical to amino acids 706-711 in SEQ ID NO: 2). All these variants with truncated parts of Aβ-43 can be made with the Aβ fragments

described herein, in particular with variants 9, 10, 11, 12, and 13.

In some cases, it is preferred that the Aβ-43 or fragments thereof are mutated. Especially preferred are substitution
5   variants where the methionine in position 35 in Aβ-43 has been substituted, preferably with leucine or isoleucine, or simply deleted. Especially preferred analogues contain one single methionine that is located in the C-terminus, either because it is naturally occurring in the amyloidogenic polypeptide or
10   foreign T_H epitope, or because it has been inserted or added. Hence, it also preferred that the part of the analogue that includes the foreign T_H epitope is free from methionine, except from the possible C-terminal location of a methionine.

In fact, it is generally preferred that all analogues of APP
15   or Aβ that are used according to the present invention share the characteristic of merely including one single methionine that is positioned as the C-terminal amino acid in the analogue and that other methionines in either the amyloidogenic polypeptide or the foreign T_H epitope are deleted
20   or substituted for another amino acid.

One further interesting mutation is a deletion or substitution of the phenylalanine in position 19 in Aβ-43, and it is especially preferred that the mutation is a substitution of this phenylalanine residue with a proline.

25   The following table sets forth a group of especially preferred constructs that operate with truncates or mutations of Aβ-43:

88

Variant No.	Aβ segment used in molecule relative to aa 1 of Aβ (1-42/43)	Position of Aβ segment relative to aa 1 of molecule	Position of P2 epitope relative to aa 1 of molecule	Position of P30 epitope relative to aa 1 of molecule	Total length of mole-cule (aa)
36	1-28	22-49	50-64	1-21	64
37	1-12 (a) + 13-28 (b)	1-12 (a) + 49-64 (b)	34-48	13-33	64
38	1-12 (x 3)	1-12, 34-45, 61-72	46-60	13-33	72
39	13-28 (x 3)	1-16, 38-53, 69-84	54-68	17-37	84
40	1-12 (a) + 13-35 (b) + 36-42 (c)	1-12 (a) + 34-56 (b) + 72-78 (c)	57-71	13-33	78
41	1-28 (x 3)	1-28, 50-77, 93-120	78-92	29-49	120
42	1-43 (F19P/M35K)	1-43	65-79	44-64	79

In this table, the Aβ segment used in the molecule is indicated
by amino acid numbers relative to aa 1 of the Aβ(1-42/43) mole-
cule, i.e. 1-28 means that fragment 1-28 of Aβ(1-42/43) is used
5   in the molecule. If two or more different segments are used,
both are indicated in the table, i.e. 1-12 (a) + 13-28 (b)
means that both fragment 1-12 and fragment 13-28 of Aβ(1-42/43)
are used in the molecule.

Also, if the same segment is present in more than one copy in
10 the construction it is indicated in the table, i.e. 1-12 (x3)
shows that fragment 1-12 of Aβ(1-42/43) is present in three
copies in the construction.

Further, the position of the Aβ segment in the molecule is
shown by amino acid positions relative to the first amino acid
15 of the molecule, i.e. 22-49 shows that the Aβ fragment in ques-
tion is positioned from amino acid 22 to amino acid 49 in the
molecule, both positions included. Positions of the P2 and P30
epitopes are indicated equivalently. If two or more different
Aβ fragments are used in the molecule, their positions are all
20 shown, i.e. 1-12 (a) + 49-64 (b) means that fragment (a) is

positioned from aa 1-12 in the molecule and fragment (b) from
aa 49-64.

Moreover, if more than one copy of the same fragment is pre-
sent in the molecule, positions for all copies are shown, i.e.
5   1-12, 34-45, 61-72 shows that the three copies of the Aβ frag-
ment are placed from position 1-12, 34-45 and 61-72, respec-
tively, in the molecule.

Finally, the total length indication of each molecule includes
both the Aβ fragment(s) and the P2 and P30 epitopes.

10 Variant 42 contains two amino acid substitutions at positions
19 (phe to pro) and 35 (met to lys) as it is indicated in the
column showing the Aβ fragments.

See Fig. 1 and the tables above for details on particular
points for introduction of the foreign T-cell epitopes.

15 One further type of construct is especially preferred. Since
one goal of the present invention is to avoid destruction of
the cells producing APP whereas removal of Aβ is desired, it
seems feasible to prepare autovaccine constructs comprising
only parts of Aβ which are not exposed to the extracellular
20 phase when present in APP. Thus, such constructs would need to
contain at least one B-cell epitope derived from the amino
acid fragment defined by amino acids 700-714 in SEQ ID NO: 2.
Since such a short polypeptide fragment is predicted to be
only weakly immunogenic it is preferred that such an autovac-
25 cine construct consists of several copies of the B-cell epi-
tope, e.g. in the form of a construct having the structure
shown in Formula I in the detailed disclosure of the present
invention, cf. above. In that version of Formula I, the terms

amyloid$_{e1}$-amyloid$_{ex}$ are x B-cell epitope containing amino acid
sequences derived from amino acids 700-714 of SEQ ID NO: 2. A
preferred alternative is the above-detailed possibility of
coupling the amyloidogenic (poly)peptide and the selected fo-
5   reign T-helper epitope to via an amide bond to a polysaccha-
ride carrier molecule – in this way multiple presentaions of
the "weak" epitope constituted by amino acids 700-714 of SEQ
ID NO: 2 become possible, and it also becomes possible to se-
lect an optimum ratio between B-cell and T-cell epitopes.

10   EXAMPLE 2

Immunisation of transgenic mice with Aβ and modified proteins
according to the invention

Construction of the hAB43+-34 encoding DNA. The hAB43+-34 gene
was constructed in several steps. First a PCR fragment was
15   generated with primers ME#801 (SEQ ID NO: 10) and ME#802 (SEQ
ID NO: 11) using primer ME#800 (SEQ ID NO: 9) as template.
ME#800 encodes the human abeta-43 fragment with E. coli opti-
mised codons. ME#801 and 802 adds appropriate restriction
sites to the fragment.

20   The PCR fragment was purified, digested with *NcoI* and *HindIII*,
purified again and cloned into *NcoI-HindIII* digested and puri-
fied pET28b+ *E. coli* expression vector. The resulting plasmid
encoding wildtype human Aβ-43 is named pAB1.

In the next step the T-helper epitope, P2, is added to the C-
25   terminus of the molecule. Primer ME#806 (SEQ ID NO: 12) con-
tains the sequence encoding the P2 epitope, thus generating a
fusion of P2 and Abeta-43 by the PCR reaction.

The cloning was performed by making a PCR fragment with pri-
mers ME#178 (SEQ ID NO: 8) and ME#806 using pAB1 as template.
The fragment was purified, digested with *NcoI* and *HindIII*, pu-
rified again and cloned into an *NcoI-HindIII* digested and pu-
5 rified pET28b+ vector. The resulting plasmid is called pAB2.

In an analogous manner, another plasmid was made harbouring
the Aβ-43 encoding sequence with another T helper epitope, P30,
added to the N-terminus. This was done by making a PCR frag-
ment with primers ME#105 (SEQ ID NO: 7) and ME#807 (SEQ ID NO:
10 13) using pAB1 as template.

The fragment was purified, digested with *NcoI* and *HindIII*, pu-
rified again and cloned into an *NcoI-HindIII* digested and pu-
rified pET28b+ vector. The resulting plasmid is called pAB3.

In the third step, a second Aβ-43 repeat is added C-terminally
15 to the P2 epitope of plasmid pAB2 by primer ME#809 (SEQ ID NO:
14). ME#809 at the same time creates a *Bam*HI site immediately
after the Aβ-43 repeat. A PCR fragment was made with primers
ME#178 and ME#809 using pAB2 as template. The fragment was di-
gested with *NcoI* and *HindIII*, purified and cloned into *NcoI-*
20 *HindIII* digested and purified pET28b+ vector. This plasmid is
named pAB4.

Finally, the P30 epitope – Aβ-43 repeat sequence from pAB3 was
cloned into pAB4 plasmid. This was done by making a PCR frag-
ment with primers ME#811 (SEQ ID NO: 16) and ME#105 using pAB3
25 as template. The fragment was purified and used as primer in a
subsequent PCR with ME#810 (SEQ ID NO: 15) using pAB3 as tem-
plate. The resulting fragment was purified, digested with
*Bam*HI and *HindIII* and cloned into *BamHI-HindIII* digested and

purified pAB4 plasmid. The resulting plasmid, pAB5, encodes the hAB43+-34 molecule.

All PCR and cloning procedures were done essentially as described by Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989
5 "Molecular cloning: a laboratory manual". 2nd. Ed. Cold Spring Harbor Laboratory, N.Y.

For all cloning procedures *E. coli* K-12 cells, strain Top-10 F' (Stratagene, USA), were used. The pET28b+ vector was purchased from Novagen, USA. All primers were synthesised at DNA
10 Technology, Denmark.

Expression and purification of hAB43+-34. The hAB43+-34 protein encoded by pAB5 was expressed in BL21-Gold (Novagen) *E. coli* cells as described by the suppliers of the pET28b+ system (Novagen).

15 The expressed hAB43+-34 protein was purified to more than 85% purity by washing of inclusion bodies followed by cation-exchange chromatography using a BioCad purification workstation (PerSeptive Biosystems, USA) in the presence of 6 M urea. The urea was hereafter removed by stepwise dialysis against a so-
20 lution containing decreasing amounts of urea. The final buffer was 10 mM Tris, pH 8.5.

Immunisation study. Mice transgenic for human APP (Alzheimer's precursor protein) were used for the study. These mice, called TgRND8+, express a mutated form of APP that results in high
25 concentration of Aβ-40 and Aβ-42 in the mouse brains (Janus, C. *et. al.*)

The mice (8-10 mice per group) were immunised with either Abeta-42 (SEQ ID NO: 2, residues 673-714, synthesised by means

of a standard Fmoc strategy) or the hAB43+-34 variant (con-
struct 34 in the table in Example 1, recombinantly produced)
four times at two-week intervals. Doses were either 100 mg for
Aβ or 50 mg for hAB43+-34. Mice were bled at day 43 (after

5 three injections) and after day 52 (after four injections) and
the sera were used to determine the level of anti-Aβ-42 spe-
cific titres using a direct Aβ-42 ELISA.

The following tabel shows the mean relative anti-Abeta-42 ti-
tres.

Immunogen	Day 43 (after 3 immunizations)	Day 52 (after 4 immunizations)
Aβ-42	4000	3000
hAB43+-34	16000	23000

10

As will be clear, the antibody titers obtained when immunizing
with the hAB43+-34 Aβ variant are approximately 4 times and 7.5
times higher after 3 and 4 immunizations, respectively, than
the titers obtained when using the unaltered wild-type Aβ-42 as

15 an immunogen. This fact is put further in perspective, when
considering the fact that the amount of variant used for immu-
nization was only 50% of the amount of wild-type sequence used
for immunization.

EXAMPLE 3

20 *Synthesis of an Aβ Peptide Copolymer Vaccine using activated
poly-hydroxypolymer as the cross-linking agent.*

Introduction. A traditional conjugate vaccine consists of a
(poly)peptide coupled covalently to a carrier protein. The
peptide contains the B-cell epitope(s) and the carrier protein

25 provides T-helper epitopes. However, most of the carrier pro-

tein will normally be irrelevant as a source for T-helper epi-
topes, since only a minor part of the total sequence contains
the relevant T-helper epitopes. Such epitopes can be defined
and synthesized as peptides of e.g. 12-15 amino acids. If

5   these peptides are linked covalently to peptides containing
the B-cell epitopes, e.g. via a multivalent activated poly-hy-
droxypolymer, a vaccine molecule that only contains the rele-
vant parts can be obtained. It is further possible to provide
a vaccine conjugate that contains an optimized ratio between

10 B-cell and T-cell epitopes.

Synthesis of the acticated poly-hydroxypolymer. Poly-hydroxy-
polymers such as dextran, starch, agarose etc. can be acti-
vated with 2,2,2-trifluoroethanesulfonyl chloride (tresyl
chloride), either by means of a homogenous synthesis (dextran)

15 dissolved in N-methylpyrrolidinone (NMP) or by means of a he-
terogeneous synthesis (starch, agarose, cross-linked dextran)
in e.g. acetone.

225 ml dry N-methyl pyrrolidinone (NMP) is added under dry con-
ditions to freeze dried, water-soluble dextran (4.5 g, 83

20 mmol, clinical grade, Mw(avg) 78000) in a 500 ml round bottom
flask supplied with a magnet for stirring. The flask is placed
in a 60°C oil bath with magnetic stirring. The temperature is
raised to 92°C over a period of 20 min. When the dextran is
dissolved the flask is immediately removed from the oil bath

25 and the temperature in the bath is lowered to 40°C. The flask
is placed into the oil bath agaom, still with magnetic stir-
ring, and tresyl chloride (2.764 ml, 25 mmol) is added drop-
wise. After 15 min, dry pyridine (anhydrous, 2.020 ml, 25 mmol)
is added drop-wise. The flask is removed from the oil bath and

30 stirred for 1 hour at room temperature. The product (Tresyl Ac-
tivated Dextran, TAD) is precipitated in 1200 ml cold ethanol

(99.9%). The supernatant is decanted and the precipitate is
harvested in 50 ml polypropylene tubes in a centrifuge at 2000
rpm. The precipitate is dissolved in 50 ml 0.5% acetic acid,
dialyzed 2 times against 5000 ml 0.5% acetic acid and freeze

5   dried. TAD can be stored as a freeze dried powder at -20°C.

An insoluble poly-hydroxypolymer, such as agarose or
croos-linked dextran can be tresyl activated by making a sus-
pension of the poly-hydroxypolymer in e.g. acetone and perform
the synthesis as a solid phase synthesis. The activated

10   poly-hydroxypolymer can be harvested by filtration. Suitable
methods are reported in e.g. Nilsson K and Mosbach K (1987),
Methods in Enzymology **135**, p. 67, and in Hermansson GT *et al.*
(1992), in "Immobilized Affinity Ligand Techniques", Academic
Press, Inc., p. 87.

15   **Synthesis of the A Beta Peptide Copolymers Vaccines**. TAD (10
mg) is dissolved in 100 μl H_2O and 1000 μl carbonate buffer, pH
9.6, containing 5 mg Aβ-42 (SEQ ID NO: 2, residues 673-714),
2.5 mg P2 (SEQ ID NO: 4) and 2.5 mg P30 (SEQ ID NO: 6) is
added. The Aβ-42 and the P2 and P30 peptides all contain pro-

20   tected lysine groups: these are in the form of 1-(4,4-Di-
methyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protected ly-
sine groups. The peptides are prepared by means of a standard
Fmoc strategy, where the conventional Fmoc-Lys(Boc)-OH has
been substituted with Fmoc-Lys(Dde)-OH (obtained from Novabio-

25   chem , cat. no. 04-12-1121), *i.e.* the ε-amino group in lysine
is protected with Dde instead of Boc.

The pH value is measured and adjusted to 9.6 using 1 M HCl.
After 2.5 hours at room temperature, hydrazine from an 80% so-
lution is added to a final hydrazine koncentration of 8% and

30   the solution is incubated for another 30 min. at room tempera-

96

ture and freeze-dried immediately hereafter. The freeze-dried product is dissolved in H_2O and dialysed extensively against H_2O before the final freeze-drying.

The ratio between B-cell epitopes (Aβ) and T-helper epitopes

5   (P2 and P30) in the final product can be varied by using different concentrations of these peptides in the synthesis step. Furhermore, the final product can be tagged with e.g. mannose (so as to target the conjugate to APCs) by adding aminated mannose to the carbonate buffer in the synthesis step.

10   If an insoluble activated poly-hydroxypolymer is used to combine the peptides containing the B-cell epitope and the T-helper epitopes, the coupling to the polymer can be performed as a solid phase synthesis and the final product is harvested and purified by wash and filtration.

15   As mentioned in the general description, the presently described approach for preparing a peptide based vaccine may be applied to any other polypeptide antigen where it would be convenient to prepare a purely synthetic peptide vaccine and where the polypeptide antigen in question provides a suffi-

20   cient immunogenicity in one single peptide:

EXAMPLE 4

Synthesis Peptide Copolymer Vaccines

TAD (10 mg) is dissolved in 100 μl H_2O and 1000 μl carbonate buffer, pH 9.6, containing 1-5 mg peptide A (any immunogenic

25   peptide of interest!), 1-5 mg P2 (diphtheria toxoid P2 epitope) and 1-5 mg P30 (diphtheria toxoid P30 epitope) is added. The pH value is measured and adjusted to 9.6 using 0.1 M HCl.

After 2.5 hours at room temperature the solution is freeze
dried immediately hereafter. The freeze-dried product is dis-
solved in H_2O and dialysed extensively against H_2O or desalted
on a gelfiltration column before the final freeze-drying. In

5 case the peptides have lysine in the sequence the ε-amine in
the lysine side chain should be protected by Dde using the
Fmoc-Lys(Dde)-OH derivative in the synthesis (Gregorius and
Theisen 2001, submitted). After coupling, hydrazine from an
80% solution is added to a final hydrazine concentration be-

10 tween 1-20% and the solution is incubated for another 30 min
at room temperature, freeze dried immediately hereafter and
dialysed extensively against H_2O or desalted on a gelfiltration
column before the final freeze-drying. The principle is set
forth in schematic form in Fig. 2.

15 Such immunogens have been utilised by the inventors with a
short C-terminal fragment of the *Borrelia burgdorferi* protein
OspC as "peptide A" and a diptheria toxoid epitope (P2 or P30)
as a peptide B. The results of immunization studies with this
antigen revealed that only the immunogen of the invention in-

20 cluding the OspC fragment and a foreign diptheria epitope
matching the MHC haplotype of the vaccinated mice were capable
of inducing antibodies reactive with OspC in these mice. In
contrast, a molecule containing only the OspC peptide was un-
able to induce antibody production and the same was true for a

25 mixture of 2 immunogens where one contained the OspC and the
other the epitope. It is therefore concluded that the inclu-
sion in the same polyhydroxypolymer carrier is superior, if
not essential, in order to induce antibody production against
a short peptide hapten as OspC.

98

LIST OF REFERENCES

Brookmeyer, R.; Gray, S.; Kawas, C. (1998). Projections of Alzheimer's Disease in the United States and the Public Health Impact of Delaying Disease Onset. American Journal of Public Health, 88(9), 1337-1342.

5   Buttini, M.; Orth, M.; Bellosta, S.; Akeefe, H.; Pitas, R.E.; Wyss-Coray, T.; Mucke, L.; Mahley, R.W. (1999). Expression of Human Apolipoprotein E3 or E4 in the Brains of Apoe-/- Mice: Isoform-Specific Effects on Neurodegeneration. Journal of Neuroscience, 19, 4867-4880.

     Clark, L.N.; Poorkaj, P.; Wszolek, Z.; Geschwind, D.H.; Nasreddine, Z.S.;
10 Miller, B.; Li, D.; Payami, H.; Awert, F.; Markopoulou, K.; Andreadis, A.; D'Souza, I.; Lee, V.M.; Reed, L.; Trojanowski, J.Q.; Zhukareva, V.; Bird, T.; Schellenberg, G.; Wilhelmsen, K.C. (1998). Pathogenic Implications of Mutations in the Tau Gene in Pallido-Ponto-Nigral Degeneration and Related Neurodegenerative Disorders Linked to Chromosome 17. Proceedings of the Na-
15 tional Academy of Sciences U.S.A., 95(22), 13103-13107.

     Gupta, R. K. et. al. (1998), Dev Biol Stand. **92**: 63-78.

     Hsiao K. et al. (1998) Transgenic mice expressing Alzheimer amyloid precursor proteins", Exp. Gerontol. 33 (7-8), 883-889

     Hutton, M.; Lendon, C.L.; Rizzu, P.; Baker, M.; Froelich, S.; Houlden, H.;
20 Pickering-Brown, S.; Chakraverty, S.; Isaacs, A.; Grover, A.; Hackett, J.; Adamson, J.; Lincoln, S.; Dickson, D.; Davies, P.; Petersen, R.C.; Stevens, M.; de Graaff, E.; Wauters, E.; van Baren, J.; Hillebrand, M.; Joosse, M.; Kwon, J.M.; Nowotny, P.; Che, L.K.; Norton, J.; Morris, J.C.; Reed, L.E.; Trojanowski, J.; Basun, H.; Lannfelt, L.; Neystat, M.; Fahn, S.; Dark, F.;
25 Tannenberg, T.; Dodd, P.; Hayward, N.; Kwok, J.B.J.; Schofield, P.R.; Andreadis, A.; Snowden, J.; Craufurd, D.; Neary, D.; Owen, F.; Oostra, B.A.; Hardy, J.; Goate, A.; van Swieten, J.; Mann, D.; Lynch, T.; Heutink, P. (1998). Association of Missense and 5'-Splice-Site Mutations in Tau with the Inherited Dementia FTDP-17. Nature, 393, 702-705.

30 Janus, C. et. al. (2000), Nature **408**: 979 - 982.

     Kas, H.S. (1997) J Microencapsul **14**: 689-711

     Leon, J.; Cheng, C.K.; Neumann, P.J. (1998). Alzheimer's Disease Care: Costs and Potential Savings. Health Affairs, 17(6), 206-216.

     Lippa C. F. et al. (1998) Ab-42 deposition precedes other changes in PS-1
35 Alzheimer's disease. Lancet 352, 1117-1118

     Luo, J.-J.; Wallace, W.; Riccioni, T.; Ingram, D.K.; Roth, G.S.; Kusiak, J.W. (1999). Death of PC12 Cells and Hippocampal Neurons Induced by Adenoviral-Mediated FAD Human Amyloid Precursor Protein Gene Expression. Journal of Neuroscience Research, 55(5), 629-642.

40 Naruse, S.; Thinakaran, G.; Luo, J.-J.; Kusiak, J.W.; Tomita, T.; Iwatsubo, T.; Qian, X.; Ginty, D.D.; Price, D.L.; Borchelt, D.R.; Wong, P.C.; Siso-

99

dia, S.S. (1998). Effects of PS1 Deficiency on Membrane Protein Trafficking in Neurons. Neuron, 21(5), 1213-1231.

National Institute on Aging Progress Report on Alzheimer's Disease, 1999, NIH Publication No. 99-4664.

5 Pietrobon, P.J. (1995), Pharm Biotechnol. 6: 347-61Poorkaj, P.; Bird, T.D.; Wijsman, E.; Nemens, E.; Garruto, R.M.; Anderson, L.; Andreadis, A.; Wiederhold, W.C.; Raskind, M.; Schellenberg, G.D. (1998). Tau Is a Candidate Gene for Chromosome 17 Frontotemporal Dementia. Annals of Neurology, 43, 815-825.

10 Schenk, D.; Barbour, R.; Dunn, W.; Gordon, G.; Grajeda, H.; Guido, T.; Hu, K.; Huang, J.; Johnson-Wood, K.; Khan, K.; Kholodenko, D.; Lee, M.; Liao, Z.; Lieberburg, I.; Motter, R.; Mutter, L.; Soriano, F.; Shopp, G.; Vasquez, N.; Vandevert, C.; Walker, S.; Wogulis, M.; Yednock, T.; Games, D.; Seubert, P. (1999). Immunization with A-beta Attenuates Alzheimer's
15 Disease-Like Pathology in the PDAPP Mouse. Nature, 400(6740), 173-177.

Shekunov, B. et. al. (1999), J. Crystal Growth 198/199: 1345 - 1351.

Spillantini, M.G.; Murrell, J.R.; Goedert, M.; Farlow, M.R.; Klug, A.; Ghetti, B. (1998). Mutation in the Tau Gene in Familial Multiple System Tauopathy with Presenile Dementia. Proceedings of the National Academy of
20 Sciences U.S.A., 95(13), 7737-7741.

Strittmatter, W.J.; Saunders, A.M.; Schmechel, D.; Pericak-Vance, M.; Enghild, J.; Salvesen, G.S.; Roses, A.D. (1993). Apolipoprotein E: High-Avidity Binding to $A\beta$ and Increased Frequency of Type 4 Allele in Late-Onset Familial Alzheimer Disease. Proceedings of the National Academy
25 of Sciences U.S.A., 90,1977-1981.

Vidal, R.; Frangione, B.; Rostagno, A.; Mead, S.; Revesz, T.; Plant, G.; Ghiso, J. (1999). A Stop-Codon Mutation in the BRI Gene Associated with Familial British Dementia. Nature, 399: 776-781.

Zheng H. (1996) "Mice deficient for the amyloid precursor protein gene.
30 Ann. N Y Acad. Sci., 777, 421-426.

York, P. (1999), PSTT 11: 430-440

CLAIMS

1. A method for *in vivo* down-regulation of amyloid precursor protein (APP) or beta amyloid (Aβ) in an animal, including a human being, the method comprising effecting presentation to
5 the animal's immune system of an immunogenically effective amount of at least one analogue of APP or Aβ that incorporates into the same molecule at least one B-cell epitope of APP and/or Aβ and at least one foreign T-helper epitope (T$_H$ epitope) so that immunization of the animal with the analogue
10 induces production of antibodies against the animal's autologous APP or Aβ, wherein the analogue

> a) is a polyamino acid that consists of at least one copy of a subsequence of residues 672-714 in SEQ ID NO: 2, wherein the foreign T$_H$ epitope is incorporated by means
15 of amino acid addition and/or insertion and/or deletion and/or substitution, wherein the subsequence is selected from the group consisting of residues 1-42, residues 1-40, residues 1-39, residues 1-35, residues 1-34, residues 1-28, residues 1-12, residues 1-5, resi-
20 dues 13-28, residues 13-35, residues 17-28, residues 25-35, residues 35-40, residues 36-42 and residues 35-42 of the amino acid sequence consisting of amino acid residues 673-714 of SEQ ID NO: 2; and/or

> b) is a polyamino acid that contains the foreign T$_H$ epi-
25 topes and a disrupted APP or Aβ sequence so that the analogue does not include any subsequence of SEQ ID NO: 2 that binds productively to MHC class II molecules initiating a T-cell response; and/or

101

c) is a polyamino acid that comprises the foreign T$_H$ epi-
tope and APP or Aβ derived amino acids, and comprises 1
single methionine residue located in the C-terminus of
the analogue, wherein other methionine residues in APP
or Aβ and in the foreign T$_H$ epitope have been substi-
tuted or deleted, and preferably have been substituted
by leucin or isoleucine; and/or

d) is a conjugate comprising a polyhydroxypolymer backbone
to which is separately coupled a polyamino acid as de-
fined in a) and/or a polyamino acid as defined in b)
and/or a polyamino acid as defined in c); and/or

e) is a conjugate comprising a polyhydroxypolymer backbone
to which is separately coupled 1) the foreign T$_H$ epi-
tope and 2) a polyamino acid selected from the group
consisting of a subsequence as defined in a), a dis-
rupted sequence of APP or Aβ as defined in b), and an
APP or Aβ derived amino acid sequence that comprises 1
single methionine residue located in the C-terminus,
wherein other methionine residues in APP or Aβ and in
the foreign T$_H$ epitope have been substituted or de-
leted, and preferably have been substituted by leucin
or isoleucine.

2. The method according to claim 1, wherein a substantial
fraction of B-cell epitopes of APP or Aβ are preserved in
the analogue and that

- at least one first moiety is introduced which effects
targeting of the analogue to an antigen presenting cell
(APC) or a B-lymphocyte, and/or

102

- at least one second moiety is introduced which stimulates the immune system, and/or

- at least one third moiety is introduced which optimizes presentation of the analogue to the immune system.

5   3. The method according to claim 2, wherein the first and/or of the second and/or of the third moiety is/are attached as side groups by covalent or non-covalent binding to suitable chemical groups in the APP or Aβ sequence.

4. The method according to any one of the preceding claims,
10   wherein the analogue comprises a fusion polypeptide.

5. The method according to any one of the preceding claims, wherein introduction of the amino acid substitution and/or deletion and/or insertion and/or addition results in a substantial preservation of the overall tertiary structure of APP or
15   Aβ.

6. The method according to any one of the preceding claims, wherein the analogue includes duplication of at least one B-cell epitope of APP or Aβ and/or introduction of a hapten.

7. The method according to any one of the preceding claims,
20   wherein the foreign T-cell epitope is immunodominant in the animal.

8. The method according to any one of the preceding claims, wherein the foreign T-cell epitope is promiscuous, such as a foreign T-cell epitope which is selected from a natural pro-
25   miscuous T-cell epitope and an artificial MHC-II binding peptide sequence.

9. The method according to claim 8, wherein the natural T-cell epitope is selected from a Tetanus toxoid epitope such as P2 or P30, a diphtheria toxoid epitope, an influenza virus hemagluttinin epitope, and a *P. falciparum* CS epitope.

10. The method according to any one of the preceding claims, wherein the analogue comprises B-cell epitopes which are not exposed to the extracellular phase when present in a cell-bound form of the precursor polypeptide Aβ.

11. The method according to any one of the preceding claims, wherein the analogue lacks at least one B-cell epitope which is exposed to the extracellular phase when present in a cell-bound form of the precursor polypeptide.

12. The method according to any one of the preceding claims, wherein the analogue comprises at most 9 consecutive amino acids of SEQ ID NO: 2., such as at most 8, at most 7, at most 6, at most 5, at most 4, and at most 3 consecutive amino acids.

13. The method according to claim 12, wherein the analogue comprises at least one subsequence of SEQ ID NO: 2 so that each such at least one subsequence of SEQ ID NO: 2 independently consists of amino acid stretches selected from the group consisting of 9 consecutive amino acids of SEQ ID NO: 2, 8 consecutive amino acids of SEQ ID NO: 2, 7 consecutive amino acids of SEQ ID NO: 2, 6 consecutive amino acids of SEQ ID NO: 2, 5 consecutive amino acids of SEQ ID NO: 2, 4 consecutive amino acids of SEQ ID NO: 2, and 3 consecutive amino acids of SEQ ID NO: 2.

14. The method according to claim 22 or 23, wherein the consecutive amino acids begin at an amino acid residue selected from the group consisting of residue 672, 673, 674, 675, 676,

677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, and 714.

15. The method according to any one of the preceding claims, wherein presentation to the immune system is effected by having at least two copies of an $A\beta$ derived fragment or the analogue covalently of non-covalently linked to a carrier molecule capable of effecting presentation of multiple copies of antigenic determinants.

16. The method according to any one of the preceding claims, variants d or e, wherein the polyamino acid and T_H epitope are attached to the polyhydroxypolymer by means of an amide bond.

17. The method according to any one of the preceding claims , variants d or e, wherein the the polyhydroxypolymer is a poly- saccharide.

18. The method according to any one of the preceding claims, wherein the analogue has been formulated with an adjuvant which facilitates breaking of autotolerance to autoantigens.

19. The method according to any one of the preceding claims, wherein an effective amount of the analogue is administered to the animal via a route selected from the parenteral route such as the intracutaneous, the subcutaneous, and the intramuscular routes; the peritoneal route; the oral route; the buccal route; the sublinqual route; the epidural route; the spinal route; the anal route; and the intracranial route.

20. The method according to claim 19, wherein the effective amount is between 0.5 µg and 2,000 µg of the analogue.

21. The method according to any one of claims 1-14, variants a-c, wherein presentation of the analogue to the immune system is effected by introducing nucleic acid(s) encoding the analogue into the animal's cells and thereby obtaining *in vivo*
5 expression by the cells of the nucleic acid(s) introduced.

22. The method according to claim 21, wherein the nucleic acid(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a
10 transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in chitin or chitosan, and DNA formulated with an adjuvant.

15 23. The method according to any one of claims 19-22, which includes at least one administration/introduction per year, such as at least 2, at least 3, at least 4, at least 6, and at least 12 administrations/introductions.

24. A method for treating and/or preventing and/or ameliora-
20 ting Alzheimer's disease or other diseases and conditions characterized by amyloid deposits, the method comprising down-regulating APP or Aβ according to the method of any one of the preceding claims to such an extent that the total amount of amyloid is decreased or that the rate of amyloid formation is
25 reduced with clinical significance.

25. An analogue of APP or Aβ which is derived from an animal APP or Aβ wherein is introduced a modification which has as a result that immunization of the animal with the analogue induces production of antibodies against the animal's autologous

APP or Aβ, and wherein the analogue is as defined in any one of claims 1-17.

26. An immunogenic composition comprising an immunogenically effective amount of an analogue according to claim 25, the composition further comprising a pharmaceutically and immunologically acceptable carrier and/or vehicle and optionally an adjuvant.

27. A nucleic acid fragment which encodes an analogue according to claim 25.

28. A vector carrying the nucleic acid fragment according to claim 27, such as a vector that is capable of autonomous replication.

29. The vector according to claim 28 which is selected from the group consisting of a plasmid, a phage, a cosmid, a mini-chromosome, and a virus.

30. The vector according to claim 28 or 29, comprising, in the 5'→3' direction and in operable linkage, a promoter for driving expression of the nucleic acid fragment according to claim 27, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment according to claim 27, and optionally a terminator.

31. The vector according to any one of claims 28-30 which, when introduced into a host cell, is capable or incapable of being integrated in the host cell genome.

32. The vector according to claim 30 or 31, wherein the pro-
moter drives expression in a eukaryotic cell and/or in a pro-
karyotic cell.

33. A transformed cell carrying the vector of any one of
5   claims 28-32, such as a transformed cell which is capable of
replicating the nucleic acid fragment according to claim 27.

34. The transformed cell according to claim 33, which is a mi-
croorganism selected from a bacterium, a yeast, a protozoan,
or a cell derived from a multicellular organism selected from
10 a fungus, an insect cell such as an S_2 or an SF cell, a plant
cell, and a mammalian cell.

35. The transformed cell according to claim 33 or 34, which
expresses the nucleic acid fragment according to claim 28,
such as a transformed cell, which secretes or carries on its
15 surface, the analogue according to claim 25.

36. The method according to any one of claims 1-14, variants
a-c, wherein presentation to the immune system is effected by
administering a non-pathogenic microorganism or virus which is
carrying a nucleic acid fragment which encodes and expresses
20 the analogue.

37. A composition for inducing production of antibodies a-
gainst amyloid, the composition comprising

-     a nucleic acid fragment according to claim 27 or a vector
      according to any one of claims 28-32, and

25 -   a pharmaceutically and immunologically acceptable carrier
      and/or vehicle and/or adjuvant.

38. A stable cell line which carries the vector according to any one of claims 28-32 and which expresses the nucleic acid fragment according to claim 27, and which optionally secretes or carries the analogue according to claim 25 on its surface.

1/2

Fig. 1

PepCoVac Synthesis

peptide A

peptide B

tresyl activated
dextran, TAD

Purification

Fig. 2

1
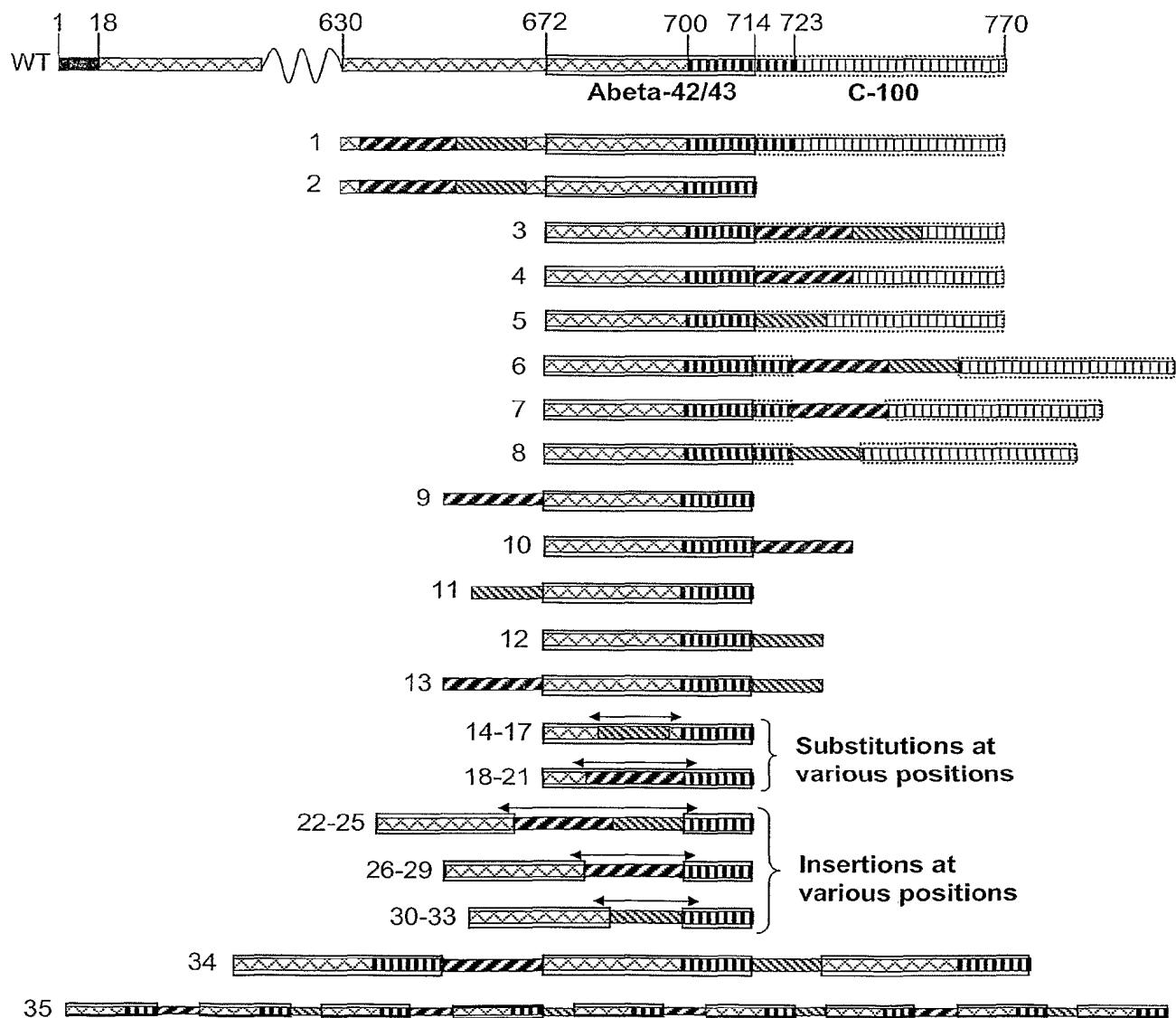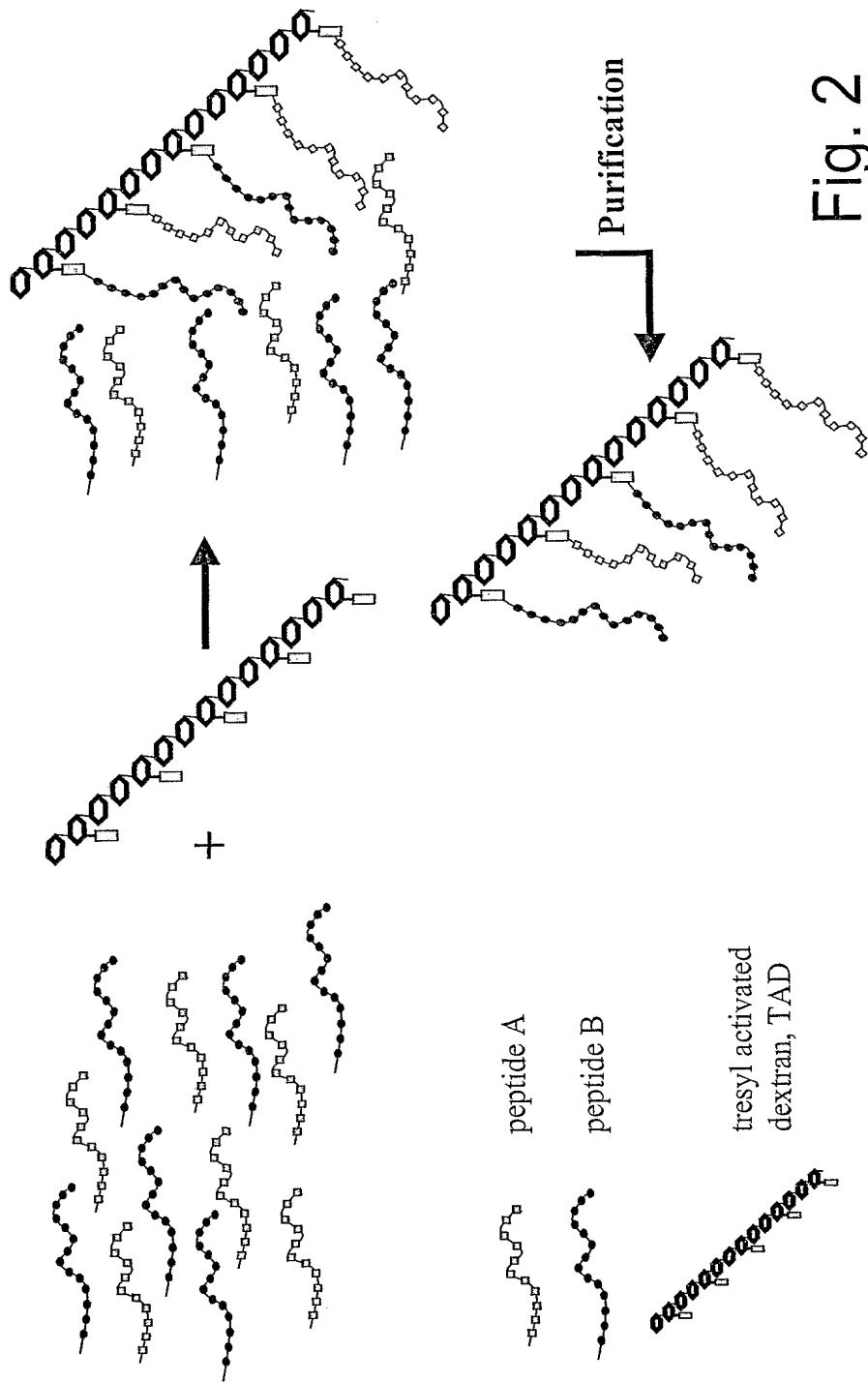
SEQUENCE LISTING

<110> Pharmexa A/S

<120> Novel Method For Down-Regulation Of Amyloid

<130> P1014PC1

<140>
<141>

<160> 16

<170> PatentIn Ver. 3.1

<210> 1
<211> 2313
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(2313)

<220>
<221> misc_feature
<222> (2098)..(2169)
<223> nucleotides encoding transmembrane region

<220>
<221> misc_feature
<222> (2014)..(2313)
<223> Nucleotides encoding C-100

<220>
<221> misc_feature
<222> (2016)..(2144)
<223> Abeta 42/43

<220>
<221> misc_feature
<222> (2014)..(2142)
<223> Abeta 42/43

<400> 1
atg ctg ccc ggt ttg gca ctg ctc ctg ctg gcc gcc tgg acg gct cgg   48
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1   5   10   15

gcg ctg gag gta ccc act gat ggt aat gct ggc ctg ctg gct gaa ccc   96
Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
                 20   25   30

cag att gcc atg ttc tgt ggc aga ctg aac atg cac atg aat gtc cag   144
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
             35   40   45

```
aat ggg aag tgg gat tca gat cca tca ggg acc aaa acc tgc att gat       192
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
        50                      55                  60

acc aag gaa ggc atc ctg cag tat tgc caa gaa gtc tac cct gaa ctg       240
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65                      70                  75                  80

cag atc acc aat gtg gta gaa gcc aac caa cca gtg acc atc cag aac       288
Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                    85                  90                  95

tgg tgc aag cgg ggc cgc aag cag tgc aag acc cat ccc cac ttt gtg       336
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                     105                 110

att ccc tac cgc tgc tta gtt ggt gag ttt gta agt gat gcc ctt ctc       384
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
            115                     120                 125

gtt cct gac aag tgc aaa ttc tta cac cag gag agg atg gat gtt tgc       432
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
        130                     135                 140

gaa act cat ctt cac tgg cac acc gtc gcc aaa gag aca tgc agt gag       480
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                     150                 155                 160

aag agt acc aac ttg cat gac tac ggc atg ttg ctg ccc tgc gga att       528
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                    165                 170                 175

gac aag ttc cga ggg gta gag ttt gtg tgt tgc cca ctg gct gaa gaa       576
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                     185                 190

agt gac aat gtg gat tct gct gat gcg gag gag gat gac tcg gat gtc       624
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
            195                     200                 205

tgg tgg ggc gga gca gac aca gac tat gca gat ggg agt gaa gac aaa       672
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
        210                     215                 220

gta gta gaa gta gca gag gag gaa gaa gtg gct gag gtg gaa gaa gaa       720
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                     230                 235                 240

gaa gcc gat gat gac gag gac gat gag gat ggt gat gag gta gag gaa       768
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                    245                 250                 255

gag gct gag gaa ccc tac gaa gaa gcc aca gag aga acc acc agc att       816
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                     265                 270

gcc acc acc acc acc acc acc aca gag tct gtg gaa gag gtg gtt cga       864
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                     280                 285
```

```
gag gtg tgc tct gaa caa gcc gag acg ggg ccg tgc cga gca atg atc    912
Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290                 295                 300

tcc cgc tgg tac ttt gat gtg act gaa ggg aag tgt gcc cca ttc ttt    960
Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305                 310                 315                 320

tac ggc gga tgt ggc ggc aac cgg aac aac ttt gac aca gaa gag tac   1008
Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
                325                 330                 335

tgc atg gcc gtg tgt ggc agc gcc atg tcc caa agt tta ctc aag act   1056
Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr
                340                 345                 350

acc cag gaa cct ctt gcc cga gat cct gtt aaa ctt cct aca aca gca   1104
Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala
            355                 360                 365

gcc agt acc cct gat gcc gtt gac aag tat ctc gag aca cct ggg gat   1152
Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
            370                 375                 380

gag aat gaa cat gcc cat ttc cag aaa gcc aaa gag agg ctt gag gcc   1200
Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
385                 390                 395                 400

aag cac cga gag aga atg tcc cag gtc atg aga gaa tgg gaa gag gca   1248
Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala
                405                 410                 415

gaa cgt caa gca aag aac ttg cct aaa gct gat aag aag gca gtt atc   1296
Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile
            420                 425                 430

cag cat ttc cag gag aaa gtg gaa ·tct ttg gaa cag gaa gca gcc aac   1344
Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn
            435                 440                 445

gag aga cag cag ctg gtg gag aca cac atg gcc aga gtg gaa gcc atg   1392
Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met
            450                 455                 460

ctc aat gac cgc cgc cgc ctg gcc ctg gag aac tac atc acc gct ctg   1440
Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu
465                 470                 475                 480

cag gct gtt cct cct cgg cct cgt cac gtg ttc aat atg cta aag aag   1488
Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys
                485                 490                 495

tat gtc cgc gca gaa cag aag gac aga cag cac acc cta aag cat ttc   1536
Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe
            500                 505                 510
```

4

```
gag cat gtg cgc atg gtg gat ccc aag aaa gcc gct cag atc cgg tcc    1584
Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser
        515                 520                 525

cag gtt atg aca cac ctc cgt gtg att tat gag cgc atg aat cag tct    1632
Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser
        530                 535                 540

ctc tcc ctg ctc tac aac gtg cct gca gtg gcc gag gag att cag gat    1680
Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp
545                 550                 555                 560

gaa gtt gat gag ctg ctt cag aaa gag caa aac tat tca gat gac gtc    1728
Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val
                565                 570                 575

ttg gcc aac atg att agt gaa cca agg atc agt tac gga aac gat gct    1776
Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala
                580                 585                 590

ctc atg cca tct ttg acc gaa acg aaa acc acc gtg gag ctc ctt ccc    1824
Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro
            595                 600                 605

gtg aat gga gag ttc agc ctg gac gat ctc cag ccg tgg cat tct ttt    1872
Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe
        610                 615                 620

ggg gct gac tct gtg cca gcc aac aca gaa aac gaa gtt gag cct gtt    1920
Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val
625                 630                 635                 640

gat gcc cgc cct gct gcc gac cga gga ctg acc act cga cca ggt tct    1968
Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser
                645                 650                 655

ggg ttg aca aat atc aag acg gag gag atc tct gaa gtg aag atg gat    2016
Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp
                660                 665                 670

gca gaa ttc cga cat gac tca gga tat gaa gtt cat cat caa aaa ttg    2064
Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
            675                 680                 685

gtg ttc ttt gca gaa gat gtg ggt tca aac aaa ggt gca atc att gga    2112
Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
        690                 695                 700

ctc atg gtg ggc ggt gtt gtc ata gcg aca gtg atc gtc atc acc ttg    2160
Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu
705                 710                 715                 720

gtg atg ctg aag aag aaa cag tac aca tcc att cat cat ggt gtg gtg    2208
Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val
                725                 730                 735

gag gtt gac gcc gct gtc acc cca gag gag cgc cac ctg tcc aag atg    2256
Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met
            740                 745                 750
```

5

cag cag aac ggc tac gaa aat cca acc tac aag ttc ttt gag cag atg   2304
Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met
        755   760   765

cag aac tag   2313
Gln Asn
    770

<210> 2
<211> 770
<212> PRT
<213> Homo sapiens

<400> 2
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1   5   10   15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
             20   25   30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
         35   40   45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
     50   55   60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65   70   75   80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                 85   90   95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100   105   110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115   120   125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130   135   140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145   150   155   160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165   170   175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180   185   190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
            195   200   205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210   215   220

6

```
Val Val Glu Val Ala Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225             230             235             240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
            245             250             255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
        260             265             270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
    275             280             285

Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
    290             295             300

Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
305             310             315             320

Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
            325             330             335

Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr
            340             345             350

Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala
        355             360             365

Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
    370             375             380

Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
385             390             395             400

Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala
            405             410             415

Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile
        420             425             430

Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn
        435             440             445

Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met
    450             455             460

Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu
465             470             475             480

Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys
            485             490             495

Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe
        500             505             510

Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser
    515             520             525

Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser
    530             535             540
```

```
Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp
545             550             555             560

Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val
            565             570             575

Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala
            580             585             590

Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro
            595             600             605

Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe
    610             615             620

Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val
625             630             635             640

Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser
            645             650             655

Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp
            660             665             670

Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
            675             680             685

Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
    690             695             700

Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu
705             710             715             720

Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val
            725             730             735

Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met
            740             745             750

Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met
            755             760             765

Gln Asn
    770
```

<210> 3
<211> 45
<212> DNA
<213> Clostridium tetani

<220>
<221> CDS
<222> (1)..(45)
<223> DNA encoding P2 epitope

8

```
<400> 3
cag tac atc aaa gct aac tcc aaa ttc atc ggt atc acc gag ctg        45
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
  1               5               10                  15


<210> 4
<211> 15
<212> PRT
<213> Clostridium tetani

<400> 4
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
  1               5               10                  15



<210> 5
<211> 63
<212> DNA
<213> Clostridium tetani

<220>
<221> CDS
<222> (1)..(63)
<223> DNA encoding P30 epitope

<400> 5
ttc aac aac ttc acc gta agc ttc tgg ctg cgt gtt ccg aaa gtt agc    48
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
  1               5               10                  15

gct agc cac ctg gaa                                                63
Ala Ser His Leu Glu
            20



<210> 6
<211> 21
<212> PRT
<213> Clostridium tetani

<400> 6
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
  1               5               10                  15

Ala Ser His Leu Glu
            20

<210>   7
<211>   21
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   7
caactcagct tcctttcggg c                                            21

<210>   8
```

```
<211>   21
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   8
agatctcgat cccgcgaaat t                                                      21

<210>   9
<211>   135
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   9
atggatgcag aattccgtca cgactccggt tacgaagttc accaccagaa actggttttc          60
ttcgcagaag atgttggttc caacaaaggt gcaatcatcg tctgatggt tggcggtgtt          120
gttatcgcga cctag                                                            135


<210>   10
<211>   31
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   10
gccggccatg gatgcagaat tccgtcacga c                                          31

<210>   11
<211>   39
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   11
gccggaagct tctaggtcgc gataacaaca ccgccaacc                                  39

<210>   12
<211>   84
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   12
ccggcaagct tctacagctc ggtgataccg atgaatttgg agttagcttt gatgtactgg          60
gtcgcgataa caacaccgcc aacc                                                   84

<210>   13
<211>   101
<212>   DNA
<213>   Synthetic
<223>   Synthetic PCR primer

<400>   13
gccggccatg ggtttcaaca acttcaccgt tagcttctgg ctgcgtgttc cgaaagttag          60
cgcgagccac ctggaagatg cagaattccg tcacgactcc g                              101

<210>   14
```

10

```
<211>  172
<212>  DNA
<213>  Synthetic
<223>  Synthetic PCR primer

<400>  14
gggccaagct tggatccggt cgcgataaca acaccgccaa ccatcagacc gatgattgca        60
cctttgttgg aaccaacatc ttctgcgaag aaaaccagtt tctggtggtg aacttcgtaa       120
ccggagtcgt gacggaactc tgcatccagc tcggtgatac cgatgaattt gg               172


<210>  15
<211>  30
<212>  DNA
<213>  Synthetic
<223>  Synthetic PCR primer

<400>  15
ctggaagatg cagagttccg tcacgactcc                                         30


<210>  16
<211>  35
<212>  DNA
<213>  Synthetic
<223>  Synthetic PCR primer

<400>  16
gcgccggatc cttcaacaac ttcaccgtta gcttc                                   35



<210>  17
<211>  13
<212>  PRT
<213>  Synthetic
<223>  Synthetic HLA DR binding sequence

<400>  17
Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
  1               5                  10
```